Lipid Oxidation Mediated by Myoglobin with Different Forms in Fish Muscle : Mode of Action and the Prevention

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ชื่อวิทยานิพนธ์	การเกิดปฏิกิริยาออกซิเดชันของไขมันในกล้ามเนื้อปลาซึ่งเหนี่ยวนำโดยไมโอโกลบินรูปแบบต่างๆ : กลไกและการป้องกัน
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สาขาวิชา	วิทยาศาสตร์และเทคโนโลยีอาหาร
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บทคัดย่อ

ไมโอโกลบินและเหล็กที่เป็นองค์ประกอบของฮีมมีปริมาณแตกต่างตามชนิดของปลา โดยปลากะพงขาวมีปริมาณสารทั้งสองชนิดสูงกว่าปลาทับทิม เมื่อทำการเก็บรักษาในน้ำแข็งเป็นเวลา 15 วัน พบว่าปลาทั้งสองชนิดมีสัดส่วนเมทไมโอโกลบินและเหล็กที่ไม่ใช่องค์ประกอบของฮีมเพิ่มขึ้น ขณะเดียวกันมีเหล็กที่เป็นองค์ประกอบของฮีมลดลง ทั้งนี้กรดไขมันไม่อิ่มตัวโดยเฉพาะอย่างยิ่ง docosahexaenoic acid (DHA) และ eicosapentaenoic acid (EPA) มีค่าลดลง ผลิตภัณฑ์จากปฏิกิริยาออกซิเดชันของไขมัน และกรดไขมันอิสระมีค่าเพิ่มขึ้น ปลาทั้งสองชนิดให้กลิ่นคาวและกลิ่นหืนเมื่อผ่านการเก็บรักษาเป็นเวลา 6 วัน โดยปลากะพงมีกลิ่นที่รุนแรงกว่าปลาทับทิม ทั้งนี้ปฏิกิริยาออกซิเดชันของไมโอโกลบินและการปลดปล่อยเหล็กจากฮีมมีความสัมพันธ์กับปฏิกิริยาออกซิเดชันของไขมันในเนื้อปลา

ไมโอโกลบินที่ผ่านการทำบริสุทธิ์จากปลาโอลายโดยการตกตะกอนด้วยแอมโมเนียมซัลเฟต (ที่ระดับความอิ่มตัว ร้อยละ 65-100) และคัดกรองขนาดด้วยโครมาโตกราฟีชนิด Sephadex G-75 มีน้ำหนักโมเลกุล 15,680 ดัลตัน ออกซีไมโอโกลบินและเมทไมโอโกลบินมีค่า pI เท่ากับ 5.25 และมีอุณหภูมิที่ก่อให้เกิดการสูญเสียสภาพธรรมชาติเท่ากับ 61 และ 60 องศาเซลเซียส ตามลำดับ ค่าสี (L^*, a^* and b^*) และสเปกตรัมการดูดกลืนแสงมีความแตกต่างกันขึ้นอยู่กับรูปแบบของไมโอโกลบิน ออกซีไมโอโกลบินและเมทไมโอโกลบินมีค่าการดูดกลืนแสงสูงสุดในช่วง Soret ที่ความยาวคลื่น 413 และ 407 นาโนเมตร ตามลำดับ ที่พีเอช 3 ไม่ปรากฏค่าการดูดกลืนแสงสูงสุดในช่วง Soret และเกิดเมทไมโอโกลบินเพิ่มขึ้น ซึ่งแสดงถึงการสูญเสียโครงสร้างของฮีมและโกลบิน และการเกิดปฏิกิริยาออกซิเดชันของไมโอโกลบิน การให้ความร้อนที่อุณหภูมิสูงกว่า 60 องศาเซลเซียส ส่งผลให้ไมโอโกลบินเกิดการสูญเสียสภาพธรรมชาติ ทั้งนี้การเพิ่มอุณหภูมิและระยะเวลาในการบ่มทำให้ออกซีไมโอโกลบินเกิดการออกซิเดชันและเปลี่ยนแปลงโครงสร้างเพิ่มขึ้น ส่วนเมทไมโอโกลบินค่อนข้างมีความคงตัว

สภาวะอิ่มตัวด้วยออกซิเจนโดยเฉพาะอย่างยิ่งเมื่อระยะเวลาเพิ่มขึ้นทำให้โครงสร้างของฮีมและโกลบินไม่คงตัวและเกิดการออกซิเดชันของโกลบิน ในสภาวะที่มีออกซิเจนทำให้ออกซีไมโอโกลบินเกิดการออกซิเดชันอย่างรวดเร็วและมีปริมาณเมทไมโอโกลบินเพิ่มขึ้น สภาวะที่ไม่มีออกซิเจนสามารถยับยั้งปฏิกิริยาออกซิเดชันของออกซีไมโอโกลบินได้ ทั้งนี้เมทไมโอโกลบินมีความคงตัวต่อการออกซิเดชันและการเปลี่ยนแปลงโครงสร้างมากกว่าออกซีไมโอโกลบิน เมื่อเก็บชิ้นปลาโอลายที่อุณหภูมิ 4 องศาเซลเซียส เป็นเวลา 3 วัน พบว่า บรรจุภัณฑ์แบบสุญญากาศทำให้กล้ามเนื้อดำของปลาโอลายมีสีแดงลดลงเพียงเล็กน้อยและยังเป็นที่ยอมรับของผู้บริโภค การเก็บภายใต้สภาวะอากาศปกติหรือสภาวะที่อิ่มตัวด้วยออกซิเจนร้อยละ 100 ทำให้ชิ้นปลาเปลี่ยนเป็นสีน้ำตาล เนื่องจากปฏิกิริยาออกซิเดชันของไมโอโกลบิน

การเติมไฮโดรเจนเปอร์ออกไซด์ (H_2O_2) ทำให้ออกซีไมโอโกลบินและเมทไมโอโกลบินเกิดปฏิกิริยาออกซิเดชันอย่างรวดเร็วกลายเป็นเฟอริลไมโอโกลบิน การเติม Fe^{2+} และ/หรือ H_2O_2 ทำให้โครงสร้างของไมโอโกลบินเกิดการเปลี่ยนแปลง โดย H_2O_2 มีบทบาทสำคัญต่อการปลดปล่อยเหล็กจากฮีม การเติมอัลดีไฮด์ทำให้ออกซีไมโอโกลบินเกิดการออกซิเดชัน และโครงสร้างโกลบินเกิดการเปลี่ยนแปลง อย่างไรก็ตามอัลดีไฮด์ไม่มีผลต่อการปลดปล่อยเหล็กจากฮีม ทั้งนี้เฮกซีนาลส่งผลให้เกิดการเชื่อมประสานของโปรตีนในออกซีไมโอโกลบินและเมทไมโอโกลบินด้วยพันธะโควาเลนต์

จากการศึกษาผลของไมโอโกลบินต่อปฏิกิริยาออกซิเดชันของไขมันในเนื้อปลากะพงบดที่ผ่านการล้างน้ำ ที่ระดับพีเอช 6.0 6.5 และ 7.0 และเก็บรักษาในตู้เย็นเป็นเวลา 8 วัน พบว่าเนื้อปลาบดที่เติมไมโอโกลบินโดยเฉพาะอย่างยิ่งที่พีเอชต่ำ มีปริมาณเมทไมโอโกลบินและการเปลี่ยนแปลงสีเพิ่มขึ้น ปฏิกิริยาออกซิเดชันของไขมัน กลิ่นคาว และกลิ่นหืนเกิดขึ้นสูงสุดที่พีเอช 6 โดยตรวจพบ 1-octen-3-ol และเฮกซานาลเป็นองค์ประกอบหลักของสารระเหยที่ให้กลิ่น

ฮีมโปรตีนที่สกัดได้จากกล้ามเนื้อดำปลาคาร์ปมีปริมาณไมโอโกลบินและฮีโมโกลบิน ร้อยละ 61 และ 39 ตามลำดับ โดยน้ำหนักโมเลกุลของไมโอโกลบินคือ 16,445 ดัลตัน ฮีโมโกลบินประกอบด้วยสายแอลฟาที่มีน้ำหนักโมเลกุลเท่ากับ 16,006 ดัลตัน และสายเบต้า 2 สายที่มีน้ำหนักโมเลกุลเท่ากับ 16,104 และ 16,180 ดัลตัน ตามลำดับ เนื้อปลาบดที่ผ่านการล้างน้ำและเติมฮีโมโกลบินมีสีแดงลดลงอย่างรวดเร็วและต่ำกว่าเนื้อปลาบดที่เติมไมโอโกลบินระหว่างการเก็บรักษาในน้ำแข็งเป็นเวลา 9 วัน ทั้งนี้เนื้อปลาบดที่เติมฮีโมโกลบินมีค่าเปอร์ออกไซด์ (PV) TBARS และเฮกซานาล สูงกว่าเนื้อปลาบดที่เติมไมโอโกลบิน โดยโปรตีนฮีมในรูปแบบเมทสามารถเร่งปฏิกิริยาออกซิเดชันของไขมันได้มากกว่าโปรตีนฮีมในรูปแบบออกซี เมื่อตรวจสอบด้วย apo Streptococcal heme-associated protein (apoShp) พบว่าฮีโมโกลบินสูญเสียฮีมจากโครงสร้างได้

มากกว่าไมโอโกลบิน ดังนั้นการปลดปล่อยฮีมและการเกิดออกซิเดชันอย่างรวดเร็วมีผลต่อกิจกรรมการเร่งปฏิกิริยาออกซิเดชันของไขมันของฮีโมโกลบิน

จากการศึกษากิจกรรมการเป็นสารต้านออกซิเดชันของไขมันซึ่งเหนี่ยวนำโดยไมโอโกลบินและฮีโมโกลบิน ของสารประกอบฟีนอลชนิดต่างๆ ได้แก่ กรดคาเฟอิก กรดแกลลิก และกรดแทนนิก ที่ระดับความเข้มข้น 200 ppm พบว่ากรดแทนนิกสามารถยับยั้งการเปลี่ยนแปลงสีของเนื้อปลาบดที่เติมไมโอโกลบินหรือฮีโมโกลบินได้ดี ทั้งนี้กรดคาเฟอิกและกรดแกลลิกสามารถยับยั้งปฏิกิริยาออกซิเดชันของไขมันได้ดีกว่ากรดแทนนิก โดยเฉพาะอย่างยิ่งที่ระยะเวลาการเก็บรักษาเพิ่มขึ้น อย่างไรก็ตามเมื่อศึกษาในชิ้นปลาโอลายที่ผ่านการแล่ พบว่ากรดแทนนิกแสดงกิจกรรมยับยั้งปฏิกิริยาออกซิเดชันของไขมันได้ดีกว่ากรดคาเฟอิก กรดแทนนิกมีฤทธิ์เสริมร่วมกับการบรรจุแบบดัดแปลงสภาพบรรยากาศ (CO_2 ร้อยละ 60/O_2 ร้อยละ 5/N_2 ร้อยละ 35) ในการยับยั้งเมทไมโอโกลบิน ปฏิกิริยาออกซิเดชันของไขมัน และการเจริญเติบโตของเชื้อจุลินทรีย์ ดังนั้นชิ้นปลาโอลายแล่จึงมีลักษณะเป็นที่ยอมรับจากผู้บริโภคระหว่างการเก็บรักษาและมีอายุการเก็บรักษานานขึ้น

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ABSTRACT

Myoglobin (Mb) and heme iron contents varied with fish species. Fresh seabass (*Lates calcarifer*) contained the higher contents of Mb and heme iron, compared with red tilapia (*Oreochromis mossambicus* × *O. niloticus*) ($P < 0.05$). During 15 days of iced storage, increases in metMb proportion and non-heme iron contents were observed in both fish, coincidentally with decreases in heme iron contents. Decreases in unsaturated fatty acids, especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), along with an increase in oxidation products and free fatty acids were observed as the storage time progressed. Fishy and rancid odors were detected at day 6 of storage for both fish and higher intensity was found in seabass muscle. Generally, oxidation of Mb and released non-heme iron were associated with lipid oxidation in fish muscle.

Mb purified from dark muscle of Eastern little tuna (*Euthynnus affinis*) by ammonium sulfate precipitation (65-100% saturation) followed by Sephadex G-75 chromatography had a molecular weight of 15,680 Da. pI of both oxyMb and metMb was estimated to be 5.25. Transition temperatures were 61 and 60ºC for oxyMb and metMb, respectively. The color values (L^*, a^* and b^*) and absorption spectra of Mb solutions differed significantly, depending upon the form of Mb. OxyMb and metMb had the Soret bands at 413 and 407 nm, respectively. Loss of heme-globin complex and autoxidation were dominant at pH 3 as evidenced by the disappearance of Soret band and formation of metMb. Heating at temperature above 60ºC had a great impact on Mb denaturation. With increasing temperature and incubation time, oxyMb was susceptible to oxidation and conformational change, while metMb tended to be more stable.

Saturated oxygen atmosphere more likely weakened heme-globin complex and oxidized globin, especially as the exposure time increased. Autoxidation of oxy-form proceeded rapidly in the presence of oxygen with the concomitant formation of met-form. When the oxygen was excluded, oxidation of oxyMb was retarded. Generally, oxyMb was more susceptible to oxidation and conformational change than did metMb. After keeping at 4ºC for 3 days, dark muscle of tuna fillet kept in vacuum packaging had a slight decrease in redness and it was still acceptable. The fillets stored in exposed air or packed in 100% O_2 atmosphere turned to be brown, most likely due to Mb oxidation.

In the presence of H_2O_2, both oxy- and metMb were rapidly oxidized into ferrylMb. Fe^{2+} and/or H_2O_2 caused the changes in conformation of Mb. Release of non-heme iron from Mb was mainly governed by H_2O_2. When aldehydes were incorporated, the oxidation of oxyMb and conformational changes of globin were more pronounced. No release of non-heme iron was noticeable, suggesting the stability of heme moiety toward aldehydes. Hexenal had a great impact on cross-linking of oxyMb and metMb via covalent modification.

The effect of Mb on lipid oxidation in washed seabass mince mediated by Mb from the dark muscle of little Eastern tuna was studied at pH 6.0, 6.5 and 7.0. During 8 days of refrigerated storage, metMb formation and discoloration increased and the changes were more pronounced at lower pH. The highest lipid oxidation and fishy and rancid off-odor development were observed in washed mince containing Mb at pH 6.0, in which 1-octen-3-ol and hexanal were the major volatile compounds.

Mb and hemoglobin (Hb) accounted for 61% and 39% of the total heme protein extracted from bighead carp (*Hypophthalmichthys nobilis*) dark muscle, respectively. Molecular weight of Mb was 16,445 Da, whereas Hb comprised two α-chains (16,006 Da) and two β-chains (16,104 and 16,180 Da). During 9 days of iced storage, loss of redness in washed mince containing Hb was more rapid and extensive, compared to that of Mb. Hb promoted the greater formation of peroxides, thiobarbituric acid-reactive substances (TBARS) and hexanal than did Mb ($P < 0.05$) and met-form was likely a stronger prooxidant than oxy-form. As determined by apo Streptococcal heme-associated protein (apoShp), heme release from Hb into apoShp was higher than that from Mb. A greater prooxidative activity of Hb was associated with low hemin affinity and rapid autoxidation.

Different phenolic compounds including caffeic acid, gallic acid and tannic acid at a level of 200 ppm were found to prevent lipid oxidation mediated by Mb and Hb. Tannic acid exhibited the retardation effect on discoloration of washed Asian carp mince containing Mb or Hb ($P < 0.05$). Caffeic acid and gallic acid generally showed the higher antioxidative activities than did tannic acid, especially at the extended storage time ($P < 0.05$). However, tannic acid had a greater preventive effect on metMb formation and lipid oxidation than did caffeic acid in Eastern little tuna slices ($P < 0.05$). Tannic acid exhibited a synergistic effect with modified atmospheric packaging (MAP: 60%CO_2/5%O_2/35%N_2) on retardation of metMb formation, lipid oxidation and microbial growth, thereby improving the acceptance and increasing the shelf-life of tuna slices during refrigerated storage.

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Yaowapa Thiansilakul

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Odor and color of raw fish are the important quality attributes for purchasing decision of consumers. High content of polyunsaturated fatty acids in fish flesh is associated with the development of undesirable odors and poor qualities due to the susceptibility to lipid oxidation. Myoglobin (Mb) has been known to be a major contributor to the color of muscle, depending on its redox state (deoxyMb, oxyMb and metMb) and concentration (Faustman and Cassens, 1990; Tang *et al.*, 2004). In general, fish Mb are more readily oxidized than the mammalian counterpart (Haard, 1992). The changes in Mb have been reported to be influenced by pH, temperature, oxygen, lipid oxidation products and the ligands (Chaijan *et al.*, 2007; Fago *et al.*, 1995; Faustman and Cassens, 1990; Faustman *et al.*, 1999). Chaijan *et al.* (2007) demonstrated that sardine Mb was prone to oxidation and denaturation at temperature above 40ºC and at very acidic or alkaline pHs. Furthermore, the rate of Mb autoxidation was related to oxygen concentration (Faustman and Cassens, 1990).

Mb has a close relationship with lipid oxidation which could influence the deterioration in food lipid-based food (Baron and Andersen, 2002; O'Grady *et al.*, 2001; Richards *et al.*, 2005). The lipid oxidation in fish muscle was promoted by autoxidation of Mb which played a considerable role in the generation of hydroperoxide (Lee *et al.*, 2003a; Sohn *et al.*, 2005). Lee *et al.* (2003a) reported that surface metMb accumulation and lipid oxidation of refrigerated tuna steaks increased during 6 days of storage, whereas sensory evaluation demonstrated the increased discoloration and the decrease in odor acceptability over time. Furthermore, hexenal and 4-hydroxynonenal, secondary products of lipid oxidation, could accelerate tuna oxyMb oxidation. This has been the basis of the general assumption that maintenance of the bright cherry-red color of meat is associated with the delay in oxidative deterioration of muscle-based foods. Richards *et al.* (2005) reported autoxidation and hemin loss of trout Mb associated with lipid oxidation in washed fish muscle at pH 6.3. Hemin loss rate was

considered more crucial in promoting lipid oxidation, compared to autoxidation rate. Released hemin readily converts pre-formed lipid hydroperoxides to radicals that facilitate lipid oxidation (Van Der Zee *et al.*, 1996).

To obtain the fish with high quality during storage, the suppression of Mb and lipid oxidation, which will in turn decrease the development of off-odor and other deteriorations, is required. Phenolic compounds, the bioactive substances widely distributed in plants, such as tea catechins, grape procyanidins, rosemary extracts and olive oil hydroxytyrosol have been reported for their antioxidative activity in fish and fish products (Pazos *et al.*, 2006; Tang *et al.*, 2001; Vareltzis *et al.*, 1997; Wang *et al.*, 2010). Furthermore, modified atmospheric packaging (MAP) has been proved to be an effective preservation method, in which the shelf-life extension and quality retention of fish and fish products can be achieved (Masniyom *et al.*, 2002). However, a little work regarding oxidative stability of fish Mb with different forms (MbFe(II) and MbFe(III)) and theirs role in accelerating lipid oxidation as well as the prevention of lipid oxidation initiated by Mb has been carried out. Therefore, it is crucial to get better understanding on a relationship between fish Mb and lipid oxidation associated with the quality changes of post-mortem fish. As a consequence, lipid oxidation and off-odor development can be prevented effectively.

1.2 Review of Literature

1.2.1 Myoglobin (Mb) in muscle tissue

The most abundant heme compounds found *in vivo* are myoglobin (Mb) and hemoglobin (Hb). Mb, the oxygen-storage protein, is a major pigment in the dark muscle of fish (Chaijan *et al.*, 2004; Stryer, 1988). Hb, which transports oxygen and other gases, is the main protein in blood and highly concentrated in the erythrocytes at above ~300 mg/ml (Jensen, 2004; Stryer, 1988). Normally, Hb contributes less to appearance of seafood than Mb because it is lost rather easily during handling and storage, while Mb is retained by the intracellular structure (Livingston and Brown, 1981). Mb is made up of a single polypeptide chain, globin, and a prosthetic heme group, an iron (II) protoporphyrin-IX complex (Pegg and Shahidi, 1997) (Figure 1), in which Hb structure is a tetramer of Mb-like monomers (Haard, 1992).

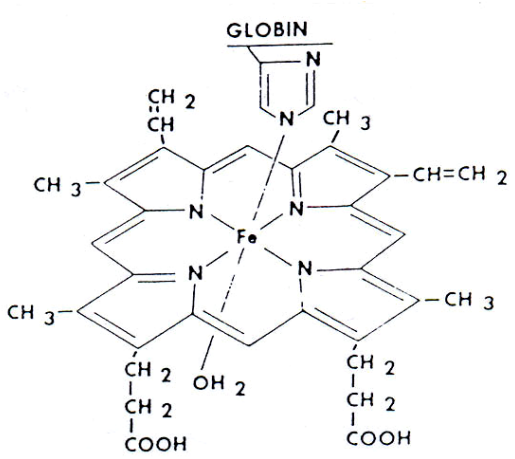


Figure 1. Chemical structure of Mb

Source: Pearson and Young (1989)

Mb polypeptide is composed of 8 separate right handed α-helices, designated A through H, that are connected by short non-helical regions. Amino acid R-groups packed into the interior of the molecule are predominantly hydrophobic in character while those exposed on the surface of the molecule are generally hydrophilic, thus making the molecule relatively water soluble (Misumi *et al.*, 2002; Pearson and Young, 1989). Globins typically consist of 145 to 155 amino acid residues, depending on species, which only three amino acid residues, CD1-Phe, E7-His and F8-His, in heme cavity are highly conserved. The heme-neighbouring CD1-Phe and iron-binding F8-His are strictly conserved in all globins. Particularly, the E7-His which is associated with ligand-binding properties, has been replaced by Gln in a few globins of vertebrate and by Val, Leu, Tyr or Gln in a considerable number of globins of invertebrate (Suzuki and Imai, 1998). Mb molecule contains one heme prosthetic group inserted into a hydrophobic cleft in the protein. Each heme residue contains one iron atom that is normally in the Fe^{2+} or ferrous (Pegg and Shahidi, 1997). The iron atom is bonded to the four nitrogens of four pyrrole groups in the center of a near-planar ring. Iron is coordinated in an octahedral environment such that it can further accept two ligands to the heme plane. These sites are occupied by an imidazole group of the histidine residue of globin and an atom possessing a free electron pair which facilitates for oxygen (Livingston and Brown, 1981). The molecular weight of Mb is determined to be 14-18 kDa as shown in Table 1. It should be noted that the molecular weight of fish Mb is generally smaller than that of mammalian Mb.

Table 1. Molecular weight of Mb from different species

Species	Molecular weight (Da)	Sources
Mackerel	14,000	Yamaguchi *et al.* (1979)
Sardine	15,300	Chaijan *et al.* (2007)
Bigeye tuna	15,540	Ueki and Ochiai (2004)
Milkfish	15,900	Chen and Chow (2001)
Yellowfin tuna	16,000	Fosmire and Brown (1976)
Sperm whale	17,000	Satterlee and Zachariah (1972)
Bovine	17,000	Renerre *et al.* (1992)
Water buffalo	17,034	Dosi *et al.* (2006)
Ovine	17,100	Satterlee and Zachariah (1972)
Porcine	17,700	Satterlee and Zachariah (1972)

The stability of Mb varies with species due to different amino acid sequences and secondary structure of globin. Generally, fish Mb is more rapidly oxidized than the mammalian Mb. Livingston and Brown (1981) and Haard (1992) reported that fish Mb are at least 2.5 times more sensitive to autoxidation than mammalian counterpart due to a cysteinyl residue at position 9 (Cys 9). African elephant Mb shows unusual autoxidation behaviour, probably due to the Gln at position 64, in place of the distal His, which is conserved in almost all Mb (Tada *et al.*, 1998). The heme-globin association or dissociation rate is generally low at neutral pH and temperature below 40ºC due to a compact and unique spatial structure with an extended hydrophobic core (Chaijan *et al.*, 2007; Chen *et al.*, 2004; Dosi *et al.*, 2006; Giddings, 1974). Under very acidic or alkaline conditions or at high temperature, the α-helical portion of Mb may be weakened and changed into a randomly coiled structure (Chaijan *et al.,* 2007; Puett, 1973). When pH is close to isoelectric point (pI), proteins often form undesirable and uncontrollable aggregates. Mackerel and sardine Mb have the pI of 5.8-5.9 (Yamaguchi *et al.*, 1979). Shiraki *et al.* (2002) reported that aggregation of horse Mb (pI = 8.1) could be prevented by addition of positively charged amino acids (Lys and Arg).

Mb exists in different redox states, as illustrated in Figure 2. Physiologically active forms of Mb are deoxyMb (MbFe(II)) and oxyMb ($MbO_2Fe(II)$). A continuous oxidation to metMb (MbFe(III)) takes place *in vivo*, though subsequent enzymatic reduction to the deoxy-forms occurs (Baron and Andersen, 2002). Interconversion among deoxyMb, oxyMb and metMb is influenced by several factors including pH, temperature, relative humidity, partial oxygen pressure (pO_2), metMb reducing activity and lipid oxidation (Faustman and Cassens, 1990). The ferric iron, unlike its ferrous counterpart, has a high nuclear charge and does not engage in strong π bonding. Therefore, metMb is unable to form an oxygen adduct (Pegg and Shahidi, 1997).

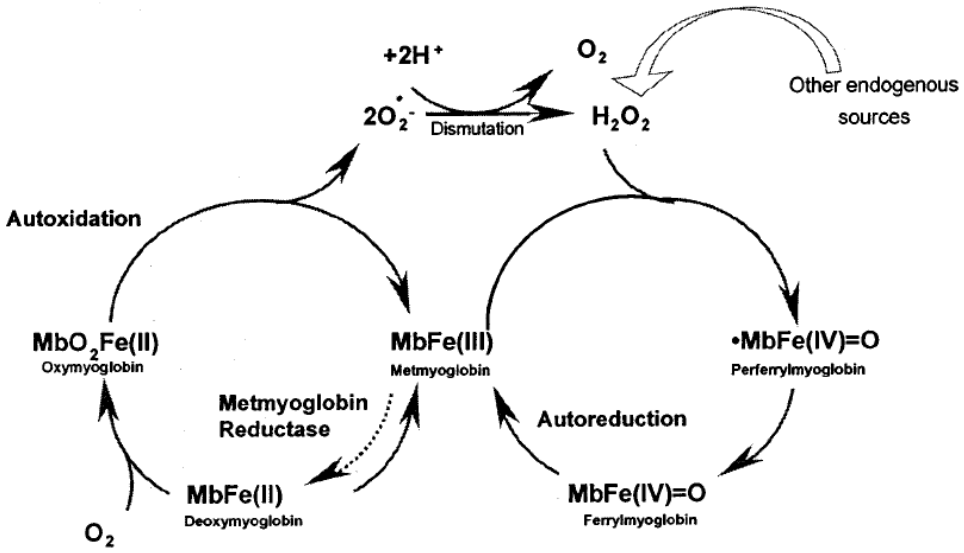


Figure 2. Dynamic conversion between the different Mbs

Source: Baron and Andersen (2002)

As shown in Figure 2, metMb can be activated by reaction with H_2O_2, resulting in the formation of an unstable hypervalent, perferrylMb ($^{\bullet}$MbFe(IV)), which is rapidly reduced to the more stable ferrylMb (MbFe(IV)) (Baron and Andersen, 2002). Even though the ferrylMb is relatively stable, it is slowly reduced to the met-form. The color of fresh meat is defined by the relative amounts of three derivatives of Mb (deoxyMb, oxyMb and metMb). Reduced Mb was the purple pigment of deep muscles and the meat under vacuum (Hanan and Shaklai, 1995). Upon exposure to air, Mb combines with oxygen to form the bright red oxyMb, which is synonymous

with freshness and is considered attractive by the consumer. However, the contact of Mb with oxygen also leads to the formation of the oxidized form, metMb, which is brown or gray and rather unattractive (Eder, 1996). Tang *et al.* (2004) demonstrated that 3 redox forms of Mb result in individually different absorption spectra which have the isobestic point at 525 nm. The maximum wavelength at 503, 557 and 582 nm represents for metMb, deoxyMb and oxyMb, respectively, as shown in Figure 3.

The Mb content is highly related to the metabolic pattern of the muscle, and its concentration varies to some extent with the types of muscle examined. The normal Mb contents of different species or age class are shown in Table 2 (Judge *et al.*, 1994). In fish muscle, Mb content varies with muscle type and species. Muscle of yellowfin tuna (*Neothunnus macropterus*) contained Mb ranging from 0.37 to 1.28 (mg/g) in light muscle and 5.30 to 24.40 (mg/g) in dark muscle (Brown, 1962). Chaijan *et al.* (2004) reported that Mb contents of ordinary and dark muscle were 2.18 and 14.27 (mg/g) for sardine (*Sardinella gibbosa*) and 1.37 and 4.88 (mg/g) for mackerel (*Rastrelliger kanagurta*), respectively.

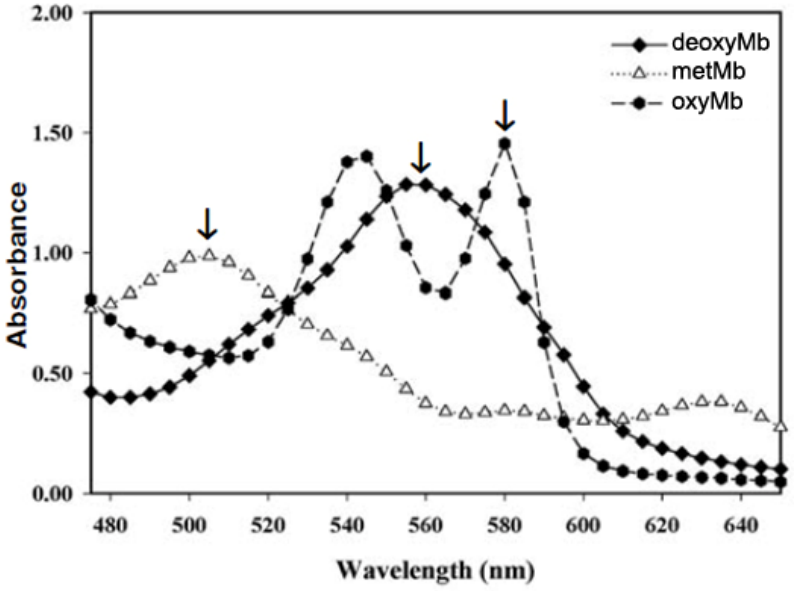


Figure 3. Absorption spectra of deoxyMb, metMb and oxyMb solutions containing equivalent Mb concentrations

Source: Tang *et al.* (2004)

Table 2. Mb contents of different species/age class

Species/Age class	Mb content (mg/g)
Pork	2
Lamb	6
Veal	2
Calf	4
Young beef	8
Old beef	18

Source: Judge *et al.* (1994)

1.2.2 Autoxidation of Mb

Oxidation state of Mb contributes to the apparent color of meat (Chaijan *et al.*, 2005; Faustman and Cassens, 1990). DeoxyMb and oxyMb are in the ferrous state (Fe(II)), whereas met form is in the ferric state (Fe(III)). The process by which ferrous Mb is converted to ferric metMb is called 'autoxidation'. Superoxide anion radical ($O_2^{\bullet-}$) or $^{\bullet}OOH$ is liberated in this process depending on whether deoxy or oxy heme protein undergoes autoxidation (Brantley *et al.*, 1993). The oxidation product of Mb, metMb, is responsible for discoloration of meat and acceleration of lipid oxidation (Baron and Andersen, 2002; Faustman and Cassens, 1990). Furthermore, ferrylMb (MbFe(IV)), cross-linked Mb and hemichrome formation, release of hemin porphyrin moiety as well as release of iron atom from the porphyrin ring occur during storage, which also result in the pro-oxidative property of Mb (Baron *et al.*, 2000; Baron and Andersen, 2002; Everse and Hsia, 1997; Richards *et al.*, 2009). Many factors have been known to be associated with autoxidation of Mb.

(1) pH : The autoxidation rate of Mb increased markedly with decreasing pH (Chaijan *et al.*, 2007; Wallace *et al.*, 1982). According to the Bohr's effect, increasing proton generally decreases the affinity of Hb for oxygen molecule, leading to a lowering of oxy-heme protein (Stryer, 1988). Livingston and Brown (1981) reported that autoxidation of Mb became greater and faster as pH decreased. The increasing autoxidation rate constant was also found in milkfish (*Chanos chanos*) Mb with decreasing pH in a range of 5.5-7.0 (Chen and Chow, 2001).

In addition, acid-denatured Mb is associated with protein unfolding and the loss in heme group (Chaijan *et al.*, 2007). The higher autoxidation of sardine Mb was found at very acidic or alkaline conditions as evidenced by the formation of metMb (Chaijan *et al.*, 2007). At neutral pH, a compact structure of Mb was obtained (Chen *et al.*, 2004). Eder (1996) found the decrease in metMb over the alkaline pHs (9-11). At high pH, the formation of hydroxyl-metMb occurred which appears to be similar to oxyMb color (Phillips Jr *et al.*, 1999). However, at extreme alkaline condition (pH 12), the formation of metMb increased, probably owing to the oxidation of oxyMb to yield metMb promoted by the high oxygen tension (Kitahara *et al.*, 1990). Susceptibility of Mb to oxidation varied with species. Kitahara *et al.* (1990) found that bigeye tuna oxyMb was much more prone to oxidation over the whole range of pH values (pH 5-12), compared with sperm whale oxyMb.

(2) Temperature : Autoxidation of Mb is enhanced as temperature increases (Livingston and Brown, 1981). Rising the temperature of fresh red meat tended to promote the autoxidation of Mb (Giddings, 1974). Chaijan *et al.* (2007) reported that the higher temperature, particularly above 40ºC, and the longer incubation time induced the metMb formation as well as the conformational changes of Mb. However, no metMb formation was noticeable at temperature lower than 20ºC. At higher temperature and longer time, unflolding of globin might occur and the aggregation of globin might be enhanced via hydrophobic interaction of these unfolded molecules (Chaijan *et al.*, 2007). When heated, tryptophan and tyrosine residues normally shielded in the folded Mb molecule become exposed and subsequently underwent aggregation. Hence, the intrinsic tryptophan fluorescent intensity can be used to monitor thermal unfolding or conformational changes of the tertiary structure (Clayton and Sawyer, 1999; Van Dael *et al.*, 1997). Copeland (1994) reported that elevated temperatures not only favor the unfolded state of a protein but also greatly increase the rate of collisions between unfolded protein molecules.

The rate of metMb formation is greater at the meat surface and is mostly arrested at storage temperature of -33ºC (Haard, 1992). At intermediate frozen storage temperatures between -5ºC and -15ºC, decreasing the storage temperature could result in an increased rate of autoxidation of yellowfin tuna Mb and beef Mb (Brown, 1962). The autoxidation rate constant between deoxyMb and oxyMb of bluefin tuna (*Thunnus thynnus*) was studied by Chow *et al.* (2004). It was pointed out that the autoxidation rate constant of deoxyMb

was higher than that of oxyMb between -6ºC and -20ºC and the highest autoxidation rate was observed at -10ºC for both forms. Benjakul and Bauer (2001) found that freeze-thaw cycles had no impact on metMb formation on catfish (*Silurus glanis* Linne) muscle. A small change in metMb of catfish fillets after the freeze-thaw process was probably due to the activity of metMb reductase, which could be released and activated. It has been known that metMb reductase is a component of red blood cells and can be found in fish muscle (Al-Shaibani *et al.*, 1977).

(3) Oxygen and oxygen consumption : Lee *et al.* (2003a) reported that the grinding process for tuna (*Thunnus albacares*) patties is likely a cause of greater surface metMb formation in patties than in steaks because of the higher surface for air exposure. Under air saturated conditions, oxyMb autoxidation could produce metMb and superoxide anion which was converted to H_2O_2 by dismutation. H_2O_2 from oxyMb autoxidation reacted rapidly with MbFe(II) or MbFe(III), resulting in the formation of ferrylMb (MbFe(IV)) (Baron and Andersen, 2002). However, at lower pO_2 values, deoxyMb concentration increased with decreasing pO_2. Tang *et al.* (2005a) reported that deoxyMb concentration began to increase when O_2 concentration decreased to 15% or lower (pO_2 = 24 mmHg at 1 atm). The rate of tuna Mb autoxidation, like that of mammals, is related to oxygen concentration. To minimize metMb formation in beef, oxygen must either be totally excluded or present at saturating levels (Faustman and Cassens, 1990). However, Haard (1992) pointed out that atmospheres enriched in carbon dioxide could promote the oxidation of oxyMb to metMb.

Efficiency in oxygen consumption of deoxy- and oxyMb plays an important role on autoxidation. Brantley *et al.* (1993) denoted that in the presence of oxygen, deoxy heme protein is susceptible to rapid autoxidation whereas fully oxygenated heme protein should be more resistant to autoxidation. Accordingly, Richards and Dettmann (2003) reported that trout Hb autoxidized much more rapidly than chicken or beef Hb due to the substantial amounts of deoxyHb in trouts. Poorly oxygenated Hb autoxidize faster than highly oxygenated Hb. The phenomenon is governed by the spin state of the iron atom inside the heme ring (Livingston and Brown, 1981). The ferrous iron atom of deoxy-heme protein (Fe^{2+}) is a 5-coordinated complex where the iron has 4 bonds to the porphyrin heme ring and 1 bond to a histidine residue of the globin. This causes the iron to be in a high spin state and hence highly susceptible to oxidation to ferric met-heme protein (Fe^{3+}). The iron atom of oxy-heme protein (Fe^{2+}) is a 6-coordinated

complex with an additional ligand to O_2, which causes the iron to be in a low spin state and less susceptible to oxidation (Richards *et al.*, 2002b).

Tissue oxygen consumption decreased with post-mortem time. Tang *et al.* (2005b) found that bovine mitochondrial respiration in a closed system resulted in decreased oxygen partial pressure (pO_2) and enhanced conversion of oxyMb to deoxyMb or metMb. Mitochondria are important subcellular organelles involved in energy metabolism. Mb and mitochondria are interrelated in living cells as Mb serves as an oxygen reservoir and oxygen transporter for mitochondria (Wittenberg and Wittenberg, 1987). In general, bovine mitochondria isolated up to 60 days post-mortem still had the capacity to consume oxygen (Tang *et al.*, 2005b). However, it was found that cod muscle had a low mitochondria content and there is no evidence for a direct interaction between mitochondria and Mb in fish during post-mortem storage (Richards and Li, 2004).

(4) Lipid oxidation products : Lipid oxidation results in a variety of secondary products which are predominantly *n*-alkanals, *trans*-2-alkenals, 4-hydroxy-*trans*-2-alkenals and malondialdehyde (Lynch and Faustman, 2000). The aldehyde products are more water-soluble than their parent compounds and could potentially interact with Mb (Chan *et al.*, 1997a). Hexanal, hexenal and 4-hydroxynonenal have been reported to enhance tuna oxyMb oxidation (Lee *et al.*, 2003a). Porcine metMb formation was greater in the presence of 4-hydroxynonenal (Lee *et al.*, 2003b). Faustman *et al.* (1999) explained that 4-hydroxynonenal covalently attached to oxyMb to cause structural alterations which would make the protein more susceptible to oxidation. Lynch and Faustman (2000) also determined the effect of aldehyde lipid oxidation products on oxyMb oxidation, metMb reduction and the catalytic activity of metMb as a lipid pro-oxidant *in vitro*. MetMb formation was greater in the presence of α-β-unsaturated aldehydes than their saturated counterparts of equivalent carbon chain length. Additionally, the oxidation products of phosphatidylcholine liposomes and the addition of known oxidation products of oleic and linoleic acid (2-octene, propanal, decanal, nonanal, hexanal, 2-nonenal and 2-heptenal) could accelerate oxyMb oxidation (Chan *et al.*, 1997a). However, there was no difference in oxyMb oxidation between the oxidized or unoxidized bovine muscle extracts containing lipid oxidation product during 3 days of storage at 4ºC. It appears that the final products of lipid oxidation, as opposed to

free-radical intermediates or lipid hydroperoxides, are not directly responsible for oxyMb oxidation (O'Grady *et al.*, 2001).

(5) Binding of ligand : The potential anions (CN^-, NO_2^-, CNS^-, F^-, Cl^- and Br^-) were found to cause oxidation of oxy heme protein to met heme protein complex (Fago *et al.*, 1995; Wallace *et al.*, 1982). The rate of autoxidation depends on the nature and amount of ligands. Anion-induced autoxidation of Hb and Mb proceeds through nucleophilic attack by anion. The final product has the anion bound to the iron in the oxidized form of the heme protein (Wallace *et al.*, 1982). In the presence of organic phosphates (ATP and ADP), binding of these ligands to specific sites on heme protein could lower the affinity of the heme groups for oxygen. The addition of ATP at pH 7.2 lowers oxygenation of trout Hb (Richards and Hultin, 2000) and snake Hb (Bonafe *et al.*, 1999). ATP affected the Hb oxygenation by the formation of the salt bridges to some amino acids at the interface in the central cavity between two β-chains (Perutz, 1990). However, the effect of ATP or ADP on autoxidation of fish Mb is slightly concerned due to the rapid breakdown of ATP and ADP in prerigor post-mortem tissue (Tang *et al.*, 2005b). After death, inosine monophosphate (IMP) is accumulated via dephosphorylation and deamination of ATP. This reaction usually goes to completion within 1 day (Surette *et al.*, 1988). Fraser *et al.* (1967) reported that ATP and ADP in cod muscle were depleted after 3 and 4 days of post-mortem storage at 0ºC, respectively.

1.2.3 Lipid oxidation

Lipid oxidation is one of the main factors limiting the quality and acceptability of meat and meat products (Chaijan *et al.*, 2006; Masniyom *et al.*, 2002; Nawar, 1996). Oxidation of lipids is accentuated in the immediate post-slaughter period, during handling, processing, storage and cooking. This process leads to discoloration, drip loss, off-odor and off-flavor development, texture defect and the production of potentially toxic compounds (Morrissey *et al.*, 1998; Richards *et al.*, 2002a).

1.2.3.1 Lipid oxidation process

The direct reaction of a lipid molecule with a molecule of oxygen, termed autoxidation, involves in oxidative deterioration of lipids. Lipid oxidation is a chain reaction that consists of initiation, propagation and termination reactions (Figure 4) (Nawar, 1996).

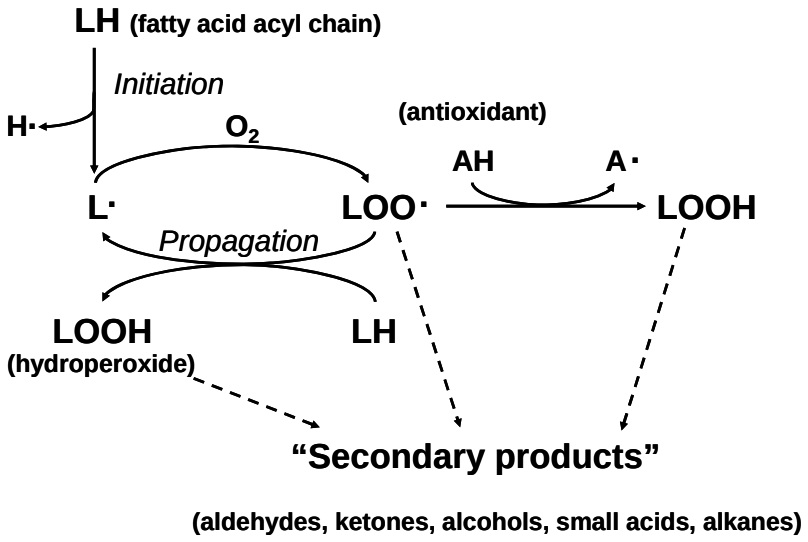


Figure 4. Autoxidation of polyunsaturated lipid

Source: Huss (1995)

Initiation: The autoxidation of lipid proceeds via typical free radical mechanisms. The process is initiated as described below by abstraction of a hydrogen atom from the central carbon of the *pentadiene structure* found in most fatty acid acyl chains (LH) containing more than one double bond:

$$\text{-CH=CH-CH}_2\text{-CH=CH-} \longrightarrow \text{-CH=CH-}^{\bullet}\text{CH-CH=CH-} + \text{H}^{\bullet}$$

The formation of lipid radical ($L^{\bullet}$) is usually mediated by trace metals, irradiation, light or heat. Also, the initiation of oxidation may take place by hydroperoxide (LOOH) decomposition, generating a highly reactive alkoxyl lipid radical ($LO^{\bullet}$) and a hydroxyl radical ($HO^{\bullet}$). Lipid hydroperoxides which exists in trace quantities prior to the oxidation can be formed by the reaction of an unsaturated fatty acid such as linoleic acid with oxygen in the singlet excited state or enzymatically by the action of lipoxygenase (Nawar, 1996). It has been postulated that singlet oxygen is the active species involved with tissue pigments such as chlorophyll and Mb acting as sensitizers. Due to resonance stabilization of lipid radical ($L^{\bullet}$) species, the reaction sequence is usually accompanied by a shift in position of the double bonds, resulting in the formation of isomeric hydroperoxides that often contain conjugated diene groups (-CH=CH-

CH=CH-) (Nawar, 1996). Conjugated diene shows a characteristic UV absorption at 232-234 nm (Nakayama, 1994).

Propagation: In propagation reaction, free radicals are converted into other radicals. Propagation of free-radical oxidation processes occurs by chain reactions that consume oxygen and yield new free-radical species (peroxy radicals, $LOO^{\bullet}$). Lipid peroxy radicals initiate a chain reaction with other molecules (LH), resulting in the formation of lipid hydropeoxides (LOOH) and lipid free radicals ($L^{\bullet}$). This reaction, when repeated many times, produces an accumulation of hydroperoxides. The propagation reaction becomes a continuous process as long as unsaturated lipid or fatty acid molecules are available. Lipid hydroperoxide, the primary products of autoxidation, are odorless and tasteless (Jadhav *et al.*, 1995).

Termination: A free radical is any atom with unpaired electron in the outermost shell. Owing to the bonding-deficiency and structural unstable, radicals therefore tend to react whenever possible to restore normal bonding. When there is a reduction in the amount of unsaturated lipids (or fatty acids) present, radicals bond to one another, forming a stable nonradical compounds. The radicals can also be removed by reaction with an antioxidant (AH) whose resulting radical ($A^{\bullet}$) is much less reactive (Huss, 1995; Jadhav *et al.*, 1995) (Figure 4).

1.2.3.2 Factors influencing lipid oxidation

The living cells possess several protection mechanisms directed against lipid oxidation products. Glutathione peroxidase reduces hydroperoxides in the cellular membranes to the corresponding hydroxy-compounds. This reaction demands supply of reduced glutathione and will therefore cease when that substance is depleted during post-mortem. The membranes also contain the phenolic compound and α-tocopherol (Vitamin E) which are considered the most important natural antioxidants (Estevez *et al.*, 2007). Tocopherol can donate a hydrogen atom to the radicals $L^{\bullet}$ or $LOO^{\bullet}$ functioning as the molecule AH as shown in Figure 4. It is generally assumed that the resulting tocopheryl radical reacts with ascorbic acid (Vitamin C) at the lipid/water interface, regenerating the tocopherol molecule. Other compounds, for example the carotenoids and catalase, are capable of function as antioxidants (Chan *et al.*, 1997b; Huss, 1995).

Many factors have been known to influence lipid oxidation.

(1) Lipid composition : In general, free fatty acid is oxidized faster than their glyceryl esters. Relatively large amounts of free fatty acid in commercial oils can increase the rate

of oxidation (Nawar, 1996). The susceptibility and rate of oxidation of fatty acids in lipids depend on the degree of their unsaturation. The autoxidation of major fatty acids of meat follows the order C18:0 < C18:1 < C18:2 < C18:3 (Shahidi, 1994). Fish, in particular, contain the higher levels of unsaturated lipids than those of mammals and birds. Thus fish lipids undergo more rapid oxidation after capture, even at low temperature storage (Foegeding *et al.*, 1996; Pacheco-Aguilar *et al.*, 2000). Pacheco-Aguilar *et al.* (2000) reported that the shelf-life of oily Monterey sardine was limited by lipid oxidation, as shown by the increase of peroxide value during storage at 0ºC up to 15 days. For Pacific saury (*Cololabis saira*), Japanese Spanish mackerel (*Scomberomorus niphonius*) and chub mackerel (*Scomber japonicus*), the higher lipid hydroperoxide contents were found in dark muscle, comparing with ordinary muscle throughout 4 days of iced storage, which positively related with the amounts of polyunsaturated fatty acids contained in the muscle (Sohn *et al.*, 2005).

(2) Pro-oxidants : Enzymes such as lipoxygenase, peroxidase and microsomal enzymes can catalytically promote lipid oxidation (Fu *et al.*, 2009; Josephson *et al.*, 1987; Slabyi and Hultin, 1982). Transition metal ions, particularly iron and copper, have been known as the major catalysts for oxidation. Tichivangana and Morrissey (1985) reported that the ferrous iron at 1-10 ppm levels acts as a strong pro-oxidant in cooked fish muscles. Castell *et al*. (1965) found that the relative pro-oxidant activity of ions in fish muscle decreased in the order of $Cu^{2+} > Fe^{2+} > Co^{2+} > Cd^{2+} > Li > Ni^{2+} > Mg^{2+} > Zn^{2+} > Ca^{2+} > Ba^{2+}$. Such metal ions either in free or bound forms occur maturely in plant and animal tissues, membranes and enzymes.

Transition metal ions in their lower valence state (M^{n+}) react very quickly with hydroperoxide. They act as one-electron donors to form an alkoxyl radical and this can be considered as the branching of the propagation step. In a slow consecutive reaction, the reduced state of the metal ion may be regenerated by hydroperoxide molecule. Furthermore, metals can abstract a hydrogen atom from the fatty acid themselves (Gordon, 2001).

$$LOOH + M^{n+} \longrightarrow LO^{\bullet} + HO^{\bullet} + M^{(n+1)+}$$

$$LOOH + M^{(n+1)+} \longrightarrow LOO^{\bullet} + H^{+} + M^{n+}$$

$$LH + M^{(n+1)+} \longrightarrow L^{\bullet} + H^{+} + M^{n+}$$

In general, dark meats tend to have more reactive iron. Chaijan *et al.* (2004) found that lipid and Mb contents were higher in dark muscle than in ordinary muscle of both sardine and mackerel. Apart from a plenty of unsaturated fatty acids, heme protein as well as reactive iron in the muscle might contribute to the accelerated oxidation (Chaijan *et al.*, 2006). Angelo *et al.* (1996) reported that iron bound to protein such as Mb, Hb and ferritin may be released during postharvest, storage and cooking, resulting in the initiation of lipid oxidation.

(3) Oxygen concentration : At very low oxygen pressure, the rate of oxidation is approximately proportional to oxygen pressure. If the supply of oxygen is unlimited, the rate of oxidation is independent of oxygen pressure. The availability of oxygen therefore plays a critical role in determining competitive oxidative pathways (Nawar, 1996). Kim *et al.* (2010) reported that oxygen at high level induced the oxidation of both lipid and Mb. The rate of oxidation increases in direct proportion to the surface area of the lipid exposed to air. Furthermore, as surface-volume ratio is increased, a given reduction in oxygen partial pressure becomes less effective in decreasing the rate of oxidation (Nawar, 1996).

(4) Temperature : Like most chemical reactions, lipid oxidation rates increase with increasing temperature and time. The highest lipid oxidation products were formed in yellowfin tuna steak stored at 21°C, followed by 4°C and 0°C, respectively (Pivarnik *et al.*, 2011). Saeed and Howell (2002) reported that the rate of lipid oxidation in frozen Atlantic mackerel increased with increasing storage time and storage temperature. Furthermore, freezing can facilitate lipid oxidation, partly because of concentration effects (Foegeding *et al.*, 1996). At elevated temperature, the increase in lipid oxidation rate affected by oxygen concentration becomes less evident, because oxygen becomes less soluble (Nawar, 1996).

(5) Salts : Organic or inorganic salts, commonly present in foods as natural components, ingredients or additive, can affect the hydrophobic/hydrophilic interactions among food components. Ionic species forming salts influence the kinetics of chemical reactions occurring in foods. NaCl is able to catalyze lipid oxidation in muscle tissue (Nambudiry, 1980). Nevertheless, Min *et al.* (2010) found that NaCl did not increase lipid oxidation products of raw chicken breast, but significantly increased those of raw beef loin patties during storage. It is most likely that meat or meat products containing salt such as surimi and cured meat are susceptible to lipid oxidation (Chaijan, 2008).

(6) Others : The extent of oxidation can be influenced by other factors such as water content, pH and emulsification. In dried food with very low moisture content (a_w < 0.1), oxidation proceeds very rapidly (Labuza *et al.*, 1972; Nawar, 1996). Increase in a_w to about 0.3 retards lipid oxidation by reducing metal catalysis, quenching free radicals, promoting nonenzymatic browning and/or impeding oxygen accessibility. At higher a_w (0.55-0.85), the rate of oxidation increases again, presumably due to increased mobilization of the catalysts (Nawar, 1996). In oil-in-water emulsions, or in foods where oil droplets are dispersed into an aqueous matrix, oxygen must gain access to the lipid by diffusion into the aqueous phase and passage through the oil-water interface. The rate of oxidation depends on the interplay between a number of factors including type and concentration of emulsifier, size of oil droplets, surface area of interface, viscosity of the aqueous phase, composition, porosity of the aqueous matrix, droplet charge of emulsifier and pH (Fomuso *et al.*, 2002; Hu *et al.*, 2004; Mei *et al.*, 1998; Nawar, 1996; Osborn and Akoh, 2004).

1.2.3.3 Lipid oxidation in fish muscle

Deterioration of lipid is one of important reactions, which limits the shelf-life of fresh fish (Chaijan *et al.*, 2006; Sohn *et al.*, 2005). Hydroperoxide, a primary oxidation product during fish storage, is readily decomposed to a variety of volatile compounds including aldehydes, ketones and alcohols, which causes the development of undesirable odors in fish flesh (Varlet *et al.*, 2006). Human olfactory receptors usually have remarkably low organoleptic thresholds to most of these volatile compounds (Ke *et al.*, 1975; McGill *et al.*, 1977). Fatty fish contains high levels of long chain n-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3). DHA and EPA are beneficial for human wholesomeness, being essential for the development and functionality of certain organs and several physiological and biochemical responses of organisms (Nawar, 1996). It has been suggested that, intake of DHA and EPA ranging from 0.5 to 1.8 g/day significantly reduces subsequent cardiac and all-cause mortality (Kris-Etherton *et al.*, 2003). Changes of lipid components as well as the loss in quality of fish, such as off-odor and off-flavor development, due to lipid oxidation are more pronounced with the extended post-mortem storage, especially under inappropriate conditions (Chaijan *et al.*, 2006; Masniyom *et al.*, 2002; Pivarnik *et al.*, 2011).

During 15 days of iced storage, EPA and DHA in sardine decreased by 19.2% and 5.9%, respectively, coincidentally with the increase in peroxide value, conjugated diene and thiobarbituric reactive substances (TBARS) throughout storage (Chaijan *et al.*, 2006). Peroxide value of horse mackerel mince increased rapidly within 24 h during storage at 5°C, together with the appearance of 1-penten-3-ol and 2, 4-heptadienal, which are typical for oxidation of n-3 fatty acids (Eymard *et al.*, 2009). Silver carp was susceptible to lipid oxidation and subsequent development of rancid and fishy odor was found during process and storage (Siddaiah *et al.*, 2001). Pivarnik *et al.* (2011) reported that loss of redness and oxidation of lipid in tuna steaks increased during room temperature, refrigerator and iced storage, while sensory evaluation demonstrated the increased deterioration of odor, texture and appearance over time. The rate of lipid oxidation of the yellowtail (*Seriola quinqueradiata*) dark muscle was significantly faster than that of the ordinary muscle and was closely related to meat darkening and development of the rancid off-odor during the early stage of iced storage (Sohn *et al.*, 2005). To evaluate the development of off-odor in raw fish due to lipid oxidation, it is important to monitor the progress of lipid oxidation in fish muscles in the early stage of storage.

1.2.4 Effect of Mb on lipid oxidation

Most biological and food studies of lipid peroxidation involve transition metal ions (Fe^{n+}, Cu^{n+}, etc.), and it is generally accepted that iron is vital in catalyzing oxidative changes in tissues (Gutteridge and Halliwell, 1990; Kanner, 1994). The concentration of ferrous iron and its ability to induce lipid oxidation reaction is a key factor causing the differences in lipid oxidation among species. It was found that iron concentration in cod muscle is very low, with an average value of 6 ppm (Vareltzis *et al.*, 2008). ‘‘Free” catalytic iron in the muscle might be generated by the destruction of the heme and release of iron. However, Undeland *et al.* (2003) suggested that the ‘‘free” iron has a negligible effect on the oxidation of washed cod muscle system. Low molecular weight iron added at a concentration of 23.2 μM did not induce oxidation of washed minced cod lipids while Hb at this concentration was very pro-oxidative (Richards and Hultin, 2000). Monahan *et al.* (1993) reported that lipid oxidation was higher in washed pork muscle treated with heme protein, compared with non-heme iron.

Under fluctuating oxygen supply and pH decrease of post-mortem system, the heme pigments like Hb and Mb become catalytic in lipid peroxidation by mechanisms involving both one- and two-electron transfer processes (Figure 5) which are different from mechanisms for lipid oxidation by the lipoxygenase (Carlsen *et al.*, 2005). Reeder and Wilson (1998) suggested that Mb plays a role as photosensitizer, which may be responsible for the initial formation of lipid hydroperoxides and increases the rate of oxygen uptake of fish oil via a photosensitized oxidation.

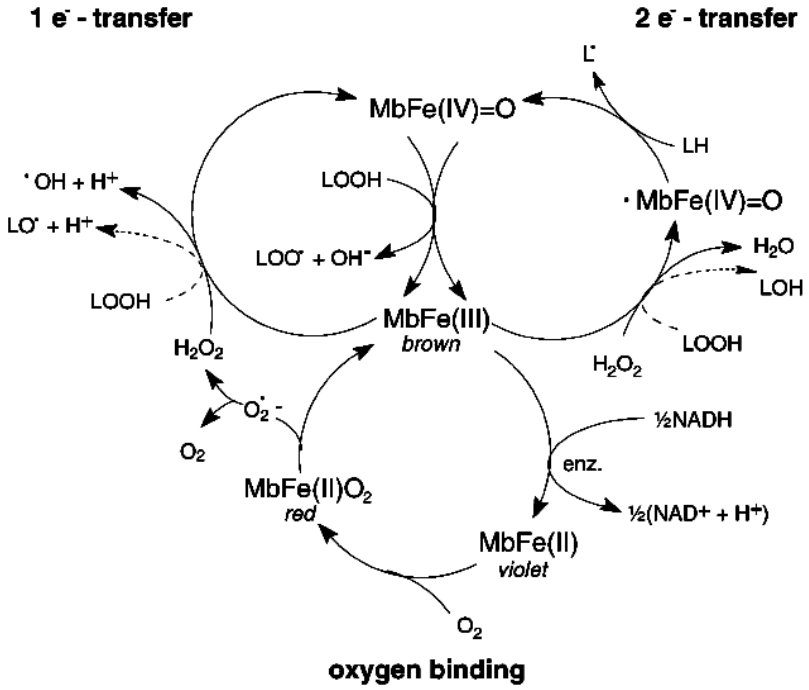


Figure 5. Mb involvement in one- and two-electron transfer processes in lipid oxidation

Source : Carlsen *et al.* (2005)

Heme-initiated lipid oxidation, especially Mb, has been extensively reported in meats (Baron and Andersen, 2002). Ohshima *et al.* (1988) and Faustman *et al.* (2010) proposed that the lipid oxidation in fish muscle was promoted by autoxidation of Mb. Moreover, O'Grady *et al.* (2001) reported a relationship between oxyMb oxidation and lipid oxidation in bovine muscle. Autoxidation of the bright red meat pigment, oxyMb, present at the surface of fresh meat, results in the formation of metmyogobin and superoxide, which rapidly dismutate to H_2O_2 and

oxygen (Baron and Andersen, 2002). Morey *et al.* (1973) found that H_2O_2, acting as an oxidizing agent, caused changes in the oxidation state of the iron in heme protein and formed red-brown in color. The interaction of H_2O_2 with metMb led very rapidly to generation of an active species, which could initiate lipid peroxidation (Chan *et al.*, 1997b; Kanner and Harel, 1985).

1.2.4.1 Iron (II) Mb-induced lipid oxidation

The physiologically active Mb species are the purple high-spin iron (II) Mb (deoxyMb), which has the sixth coordination site of the heme iron vacant, and the bright cherry-red low-spin oxy-iron (II) Mb (oxyMb), which bind a molecule of oxygen at the sixth coordination of the heme iron, due to their high affinity for oxygen (Baron and Andersen, 2002; Faustman *et al.*, 1999; Gorelik and Kanner, 2001). Disturbance of the globin structure can result in binding of the unusual ligands (e.g., the distal histidine in the heme cavity, exogenous amino acids as histidine and methionine, or a hydroxyl group) at the sixth coordination of the heme iron and induce the formation of a low-spin iron (II) species, known as hemochromes. Hemochromes in its oxidation state II can be found either reversible (binding to the imidazole group of the distal histidine or hydroxyl ion) or irreversible (binding to the imidazole group of free histidine) (Baron and Andersen, 2002).

The pro-oxidative activity of deoxyMb in biological system including muscle foods has not been investigated (Baron and Andersen, 2002). This is mainly due to the fact that deoxyMb initiated lipid oxidation demands strictly anaerobic condition; to exclude oxyMb initiated lipid oxidation and the subsequent propagation of lipid oxidation. However, Richards and Dettmann (2003) postulated that perch and trout deoxyHb could stimulate lipid oxidation in washed cod muscle during storage at 4ºC as evidenced by the formation of lipid peroxides and TBARS. A more rapid formation of metHb from deoxygenated molecules, deoxyHb, likely increases the lipid oxidation (Richards *et al.*, 2002b). Hogg *et al.* (1994) showed that oxyMb can promote oxidative modification of low density lipoprotein. Galaris *et al.* (1990) showed visible absorption spectral change of oxyMb upon incubation with linoleic acid at physiological pH. This could be attributed to the formation of the noncatalytic low-spin Mb derivative, hemochrome (Akhrem *et al.*, 1989).

Additionally, pro-oxidative activity of oxyMb is difficult to assess because of continuous autoxidation of oxyMb to metMb. Chan *et al.* (1997a) and Yin and Faustman (1993)

reported a high correlation between oxyMb oxidation and lipid oxidation both in microsomes and liposomes system. Oxidation of oxyMb plays an important role for lipid oxidation (Chan *et al.*, 1997b). Pro-oxidative activity of oxyMb in a Mb-liposome system was concentration-dependent and showed the higher activity than did metMb. The added sperm whale Mb was found to promote lipid oxidation in washed cod by which lipid oxidation occurred more rapidly at pH 5.7 compared to pH 6.3 (Grunwald and Richards, 2006a).

Apart from metMb, superoxide anion ($O_2^{\bullet -}$) and H_2O_2 are produced during oxidation of oxyMb (Gotoh and Shikama, 1976):

$$2\text{oxyMb} \longrightarrow 2\text{metMb} + O_2^{\bullet -}$$

$$2O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Superoxide anion by itself is not considered as an active catalyst of lipid oxidation, but can further dismutate to H_2O_2 (Chan *et al.*, 1997b). The generated H_2O_2, is considered as an active pro-oxidant via the Fenton's reaction to produce hydroxyl radical (Fenton, 1894):

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^-$$

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + OOH^{\bullet} + H^+$$

The hydroxyl radical has the ability to penetrate into the hydrophobic lipid region and hence facilitates lipid oxidation. Catalase, which catabolizes H_2O_2 into H_2O and O_2, was effective in inhibiting both lipid and oxyMb oxidation in an oxyMb-liposome system (Chan *et al.*, 1997b).

1.2.4.2 Iron (III) Mb-induced lipid oxidation

High-spin iron (III) Mb, commonly known as metMb, binds a molecule of water at the sixth coordination site of the heme iron (Pegg and Shahidi, 1997). Like hemochromes, the low-spin iron (III) Mb species known as hemichromes can be formed by disturbance of the globin structure. Hemichrome formation is either reversible or irreversible depending on the type of ligand at the sixth coordination site of the iron and the extent of globin denaturation. Hemichrome formation from iron (III) Mb is the intermediate step in the heat denaturation of Mb in muscle foods (Baron and Andersen, 2002). Post-mortem processes, especially the pH fall, continuously inactivate the reductive enzyme systems and stimulate acid-catalyzed autoxidation of the iron (II) states to the iron (III) state of Mb, resulting in the accumulation of metMb in meats (George and Stratmann, 1954; Gotoh and Shikama, 1976).

Formation of metMb is highly correlated to the extent of lipid oxidation in muscle foods as shown in Figure 5 (Andersen and Skibsted, 1991; Greene and Price, 1975). Meat pigments, both Fe^{2+} and Fe^{3+} forms, could catalyze lipid oxidation, but the conversion to the Fe^{3+} state was necessary for rapid catalysis. MetHb is considered more pro-oxidative than reduced Hb due to its less tightly bound heme group (Richards and Li, 2004). The result of Grunwald and Richards (2006b) also confirmed that sperm whale metMb caused a more rapid formation of lipid peroxides and TBARS in washed cod muscle as compared to ferrous Mb during 2ºC storage. MetMb is an effective pro-oxidant in the presence of H_2O_2 due to the generation of perferrylMb (Baron *et al.*, 1997). The lack of pro-oxidative activity of metMb found in linoleate emulsion systems at physiological pH has been proposed to be a result of an interaction between metMb and free fatty acids to the noncatalytic hemichrome (green pigment) (Baron *et al.*, 2000). Hemichrome, with the distal histidine reversibly bound to the iron center, is ineffective in initiating lipid peroxidation and cannot be activated to perferrylMb by addition of moderate amounts of H_2O_2 (Figure 6) (Baron *et al.*, 2000). A similar interaction between free fatty acids and metMb has been suggested (Mikkelsen and Skibsted, 1995; Nakamura and Nishida, 1971). However, only linoleate was found to associate and affect structural changes in metMb (Baron *et al.*, 2000).

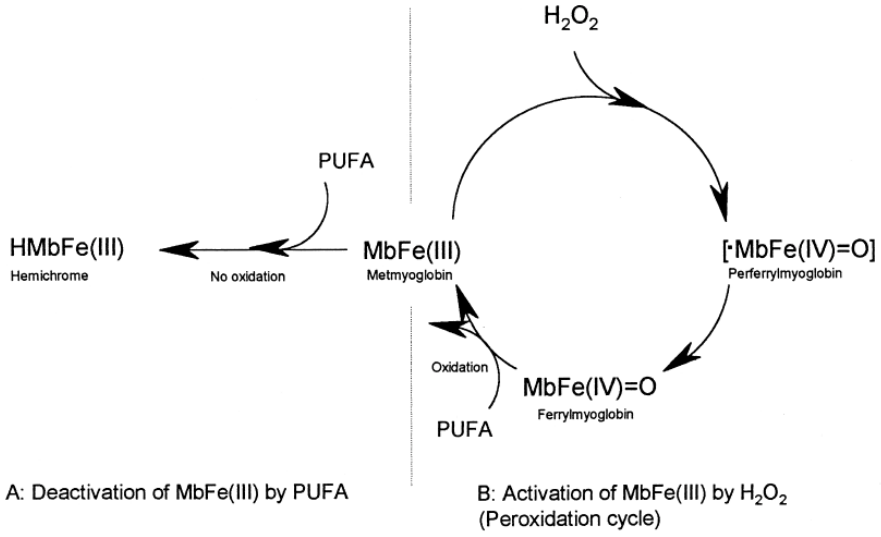


Figure 6. Proposed mechanism for reversible binding of linoleate to metMb

Source: Baron *et al.* (2000)

The lipid to heme protein ratio has been demonstrated to be an important factor affecting the pro-oxidative activity of heme proteins (Kendrick and Watts, 1969). Immediately after slaughter, the amount of free fatty acids in muscle is low, but it increases slowly during storage, reaching ~1% in 5-7 days and being most pronounced in oxidative muscles (Currie and Wolfe, 1977). Variability in the amount of free fatty acid in muscle-based foods might contribute to conflicting reports regarding the abilities of heme protein to initiate lipid oxidation in muscle foods (Kanner *et al.*, 1988). The mechanism responsible for the inhibition of lipid peroxidation at low linoleate-to-heme ratios has been demonstrated. Fatty acid anions bind reversibly to metMb, yielding the low-spin hemichrome, which was not a pro-oxidant. At high linoleate-to-heme ratios, lipid peroxidation induced by metMb or metMb/H_2O_2 was accompanied by a nearly complete or even total disappearance of the Soret absorption band (the strong absorption of Mb located in the blue region (350-450 nm)), indicating the destruction of the heme protein during incubation (Baron *et al.*, 2000). Denaturation of the heme proteins due to a high lipophilic environment results in heme release or further exposure of the heme group to the surrounding lipids, interpreted as hematin-induced lipid peroxidation (Baron and Andersen, 2002).

Heme, hematin and hemin are normally used interchangeably to describe the existence of non-protein bound heme-iron (or 'free heme iron'). Heme in solution is mainly found as hematin (ferriprotoporphyrin hydroxide). Hemin is ferriprotoporphyrin chloride which readily converts to hematin in aqueous solution and accordingly the term hematin should be used for non-protein bound heme-iron (Carlsen *et al.*, 2005). Grunwald and Richards (2006a) suggested that sperm whale Mb, having a more rapid hemin loss rate, possessed a more effective pro-oxidative activity than did modified Mb with high hemin affinity. It was found that hemin concentrations in mackerel light muscle increased around 3-fold during iced storage (Decker and Hultin, 1990). Following release of hemin from the globin, hemin is proposed to attach with phospholipids membranes due to hydrophobic attractions. Also, the propionate groups ($C_2H_5COO^-$) of hemin can bind with phospholipid headgroup amines by electrostatic interactions (Cannon *et al.*, 1984). Hemin can react with lipid hydroperoxide to form alkoxyl radical and ferryl-hydroxo complex (reaction 1) (Dix and Marnett, 1985). Ferryl-hydroxo complex can react with another lipid hydroperoxide to form a peroxyl radical and regenerate hemin (reaction 2):

$$\text{hemin(3+)} + \text{LOOH} \longrightarrow \text{LO}^{\bullet} + \text{hemin(4+)-OH} \quad \text{(reaction 1)}$$

$$\text{hemin(4+)-OH} + \text{LOOH} \longrightarrow \text{LOO}^{\bullet} + \text{hemin(3+)} + H_2O \quad \text{(reaction 2)}$$

Alkoxyl and peroxyl radicals are capable of abstracting a hydrogen atom from a polyunsaturated fatty acid which will stimulate the lipid oxidation processes (Grunwald and Richards, 2006a).

1.2.4.3 Iron (IV) Mb-induced lipid oxidation

H_2O_2 activation of metMb (also called activated-Mb) was a necessary step in the conversion of metMb to a pro-oxidant, ferrylMb (Figure 2) (Kanner and Harel, 1985). The interaction between metMb and H_2O_2 is a complex mechanism, resulting in the generation of hypervalent Mb species, perferrylMb ($^{\bullet}$MbFe(IV)=O) which is rapidly converted to the more stable ferrylMb species (MbFe(IV)=O) (Harel and Kanner, 1985; Kanner and Harel, 1985). During this interaction, the production of free radicals was postulated to occur in the globin part of the heme protein (Davies, 1990). The iron (II) Mb species, deoxyMb and oxyMb, can likewise react with H_2O_2, resulting in the formation of ferrylMb by direct two-electron oxidation of these iron (II) Mb species (Davies, 1991):

$$\text{MbFe(II)}O_2 \longrightarrow \text{MbFe(II)} + O_2$$

$$\text{MbFe(II)} + H_2O_2 \longrightarrow \text{MbFe(IV)=O} + H_2O$$

Both of hypervalent heme pigments effectively oxidize a variety of biological components (Galaris *et al.*, 1990; Newman *et al.*, 1991; Rice *et al.*, 1983). This makes it obvious to include these heme proteins in the investigation of the role of individual Mbs in oxidative deterioration of muscle-based foods (Baron and Andersen, 2002; Kanner and Harel, 1985).

PerferrylMb: Even a transient species, perferrylMb can effectively transfer its radical to other proteins, which may subsequently induce lipid oxidation (Reeder and Wilson, 1998). The ability of perferrylMb to initiate lipid oxidation and to abstract an allylic hydrogen atom from fatty acids (LH) was suggested by Kanner and Harel (1985) as follows:

$$^{\bullet}\text{MbFe(IV)=O} + \text{LH} \longrightarrow \text{MbFe(IV)=O} + \text{L}^{\bullet} + H^{+}$$

However, this applies only if MbFe(III) is activated by peroxides to yield $^{\bullet}$MbFe(IV)=O prior to interaction with the lipid components.

FerrylMb: FerrylMb has been reported to be a strong pro-oxidant of the various substances including proteins, ascorbic acids, tocopherols, glutathione, β-carotene and Trolox and to be able to initiate lipid oxidation in muscle foods (Baron and Andersen, 2002; Hogg *et al.*, 1994). The oxidation of phosphatidylcholine by ferrylMb was found to be approximately seven-fold greater than that observed for metMb (Vuletich *et al.*, 2000). FerrylMb is a relatively stable species which is slowly reduced to metMb at physiological pH but with an increasing rate at decreasing pH due to an acid-catalyzed process (Mikkelsen and Skibsted, 1995). However, under the conditions found in fresh meat (pH 5.5-5.8), ferrylMb autoreduces rapidly to metMb, and it can not be excluded that metMb plays an important role in lipid oxidation. Nevertheless, under physiological conditions (pH 7.4), ferrylMb has also been shown to initiate lipid oxidation under conditions where metMb is not a pro-oxidant (Baron and Andersen, 2002). Rao *et al.* (1994) reported that under physiological conditions, ferrylMb is a strong pro-oxidant, able to abstract a hydrogen atom from fatty acids with subsequent stereospecific addition of oxygen.

The pro-oxidative activity of ferrylMb is independent of pH and lipid concentration (Baron and Andersen, 2002). Under physiological conditions and in the presence of fatty acids, ferrylMb is not converted to non pro-oxidative hemichrome (Baron *et al.*, 2000). Increasing the lipid/heme protein ratio did not affect the pro-oxidative activity of ferrylMb (Baron *et al.*, 2002). FerrylMb is expected to be an effective pro-oxidant under the conditions found in muscle food, as well as under physiological conditions. Its potential to oxidize lipids is dependent on H_2O_2, lipid hydroperoxide production, the concentration of reducing agents and their compartmentalization in the muscle cells (Baron and Andersen, 2002).

1.2.5 Prevention of lipid oxidation and off-odor in post-mortem fish

Odor is a very complex attribute of meat palatability. Rancid or fishy odor has been identified as a common off-flavor associated with fish flesh and directly related with the formation of the secondary lipid oxidation products (Ke *et al.*, 1975; McGill *et al.*, 1977; Sohn *et al.*, 2005). Varlet *et al.* (2006) reported that carbonyl compounds, such as heptanal or (*E,Z*)-2,6-nonadienal, show a high detection frequency and odorant intensity in salmon (*Salmo salar*), giving the flesh its typical fishy odor. The fishy volatiles identified in the boiled sardine were

dimethyl sulfide, acetaldehyde, propionaldehyde, butyraldehyde, 2-ethylfuran, valeraldehyde, 2,3-pentanedione, hexanal and 1-penten-3-ol (Kasahara and Osawa, 1998).

The increased lipid hydroperoxide content and TBARS of the yellowtail dark muscle were accompanied with the increasing intensity of fishy, spoiled and rancid off-odor smells as well as increasing metMb formation. However, no correlation was found between the content of total lipid hydroperoxide and the odor intensities in ordinary muscle (Sohn *et al.*, 2005). It is believed that the formation of metMb by the oxidation of Mb predominantly in dark muscle accelerates lipid oxidation and leads to the generation of greater amounts of hydroperoxide. Thus, the lipid oxidation associated with metMb formation may have caused the development of the rancid off-odor and fishy smell in dark muscle. For ordinary muscle of yellowtail which contained a low level of metMb, the influence of Mb oxidation on the development of rancid off-odor appeared to be insignificant (Sohn *et al.*, 2005). Fu *et al.* (2009) reported that lipoxygenase caused the faster lipid oxidation in the initial phase, compared with Hb. The lipoxygenase was affiliated with strong fishy odor, while Hb resulted in severe oxidized oil odor (Fu *et al.*, 2009).

The suppression of Mb oxidation will in turn decrease lipid oxidation and off-odor development of fish muscle. Masking is one technique that has been used to reduce the sensations of aversive odors and flavors in foods. Various methods including soaking into soybean milk, adding anchovy and adding spices for masking fish odor into the extracts from salmon frame were examined by Heu *et al.* (2008). Soaking of salmon frame into soybean milk was the most efficient method for masking fish odor in the extracts from salmon frame. Fresh green leaves of perilla are often used in Japanese cuisine for masking fishy odor and as a garnish (Kasahara and Osawa, 1998). The combination effect of perilla and young leaf of Japanese pepper had the highest suppressing effect on the fishy odor as shown by sensory evaluation, gas chromatography and gas chromatography-mass spectrometry (Kasahara and Osawa, 1998).

1.2.5.1 Use of antioxidants

Antioxidant is capable of delaying, retarding or preventing the development of rancidity or other flavor deterioration due to oxidation (Gordon, 2001). The biological antioxidant, α-tocopherol, has been used both endogenously and exogenously to delay the oxidation of oxyMb and lipid in meat by neutralizing free radicals and terminating free radical

chain propagation (Lee *et al.*, 2003b). In general, antioxidants function by reducing the rate of initiation reaction in the free-radical chain reactions and are functional at very low concentrations (Gordon, 2001; Nawar, 1996).

The use of antioxidants in food products is controlled by laws and regulations of the country or by international standards. Even though many natural and synthetic compounds have antioxidant properties, only a few of them have been accepted as 'generally recognized as safe' (GRAS) substances for use in food products by international bodies such as the Joint FAO/WHO Expert Committee for Food Additives (JECFA) and the European Community's Scientific Committee for Food (SCF).

Phenolic compounds: Phenolic compounds, also denoted polyphenols, are defined as compounds possessing one or more aromatic rings bearing hydroxyl substituent(s), which are derived from the secondary metabolite of plants (Parr and Bolwell, 2000; Robards *et al.*, 1999). Plant phenolic compounds include flavonoid compounds, cinnamic acid derivatives, coumarins, tocopherols and polyfunctional organic acid (Pratt and Hudson, 1990). The reducing capacity or ability for donating electrons and the chelating properties contribute to the antioxidant activity of phenolic compounds (Medina *et al.*, 2007; Pazos *et al.*, 2006).

Horse mackerel pre-soaked with a mixture of hyssop (*Hysoppus officinalis*), brunella (*Prunella vulgaris*), lemon balm (*Melissa officinalis*) and rosemary (*Rosmarinus officinalis*) exhibited the better odor and color acceptance during frozen storage (Lugasi *et al.*, 2007). Varying structure and number of hydroxyl groups of phenolic compounds bring about the different antioxidative property. Furthermore, the anitioxidative activity generally increased with increasing phenolic compounds content. Huang and Frankel (1997) reported the antioxidative efficiency of isolated catechins from green tea leaves. The extracts included (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg), (-)-epigallocatechin gallate (EGCg). The activity of catechins in model systems was in the order of EC<ECg<EGC<EGCg. At similar molar concentrations, the activity of these compounds was superior to those of BHA and α-tocopherol in lard (Namiki, 1990). Tang *et al.* (2001) reported that antioxidant potential of tea catechins added to mackerel and whiting mince was two-fold greater than that of α-tocopherol at the same concentration (300 ppm). Corresponding to the ability of electron donating, caffeic acid proposed the most preventive effect on lipid oxidation in minced horse mackerel, compared with

the other hydroxycinnamic acids and catechins. There was an increment of antioxidant efficacy in fish muscle using concentrations ranging between 10 and 100 ppm of both caffeic acid and catechin (Medina *et al.*, 2007).

White grape dietary fiber showed the antioxidative activity against Hb-mediated oxidation of washed cod mince, by which lipid oxidation products and rancid odor were lowered and the redness was maintained (Sanchez-Alonso *et al.*, 2007). Inhibition of Hb- and iron-promoted oxidation in fish microsomes by phenolic compounds have been studied by Pazos *et al.* (2006). Isolated grape procyanidins expressed the highest antioxidative activity followed by propyl gallate, grape phenolic extract and olive oil hydroxytyrosol, respectively. Lee *et al.* (2006) reported that oligomeric polyphenols (e.g., proanthocyanidins) from cranberry extracts were least effective to inhibit Hb-mediated lipid oxidation in washed fish muscle compared to the other classes of polyphenolics (e.g., phenolic acids, flavonols and anthocyanins).

1.2.5.2 Use of modified atmosphere packaging (MAP)

Modified atmosphere packaging (MAP) is another effective preservation method, which can extend the shelf-life of fish and fish products (Masniyom *et al.*, 2002). Spoilage of fish and shellfish results from changes caused by oxidation of lipids, activities of the fish enzymes and the metabolic activities of microorganisms (Ashie *et al.*, 1996). Normally, fish stored in modified atmosphere had an equal or better sensory quality in comparison with iced storage (Sivertsvik *et al.*, 1999). The rapid growth of MAP technology for preservation of fish products is due to a number of interrelated factors such as (1) developments on new polymeric high-barrier packaging materials; (2) extended market areas for products with fresh characteristics; (3) consumer concerns about preservation additives in such products; and (4) favorable consumer perception of MAP technology (Ashie *et al.*, 1996). Several methods can be used to modify atmosphere such as vacuum packaging, gas packaging and atmosphere modifier (Ashie *et al.*, 1996; Sivertsvik *et al.*, 2002).

Vacuum packaging is used extensively for the shelf-life extension and keeping quality of fish and processed fish. The product is placed in a low oxygen permeability package. Air is removed under vacuum and the package is sealed. Under condition of a good vacuum, headspace O_2 is reduced to less than 1%, while CO_2 produced from tissue and microbial respiration eventually increases to 10-20% within the package headspace. This condition, that is

low O_2 and elevated CO_2 levels, extends the shelf-life of fresh fish by inhibition the growth of aerobic spoilage microorganisms, particularly *Psuedomonas* spp. and *Aeromonas* spp. (Ashie *et al.*, 1996).

MAP with a high level of oxygen (70-80%) is used to preserve the bright red color of meat. However, High oxygen induced oxidation of lipid and Mb as well as aggregation of myosin (Kim *et al.*, 2010). MAP under a high level of CO_2 (40-100%) is used to inhibit bacterial growth and extend shelf-life of fish and fishery products (Sivertsvik *et al.*, 2002). Additionally, the benefit of low oxygen packaging is to reduce flavor degradation due to oxidative rancidity.

There was no difference in oxyMb content of minced beef stored up to 4 days in MAP containing 20, 40, 60 or 80% oxygen. After 7 days of storage, oxyMb decreased with decreasing oxygen level, in which lipid oxidation was high in MAP containing 40, 60 or 80% oxygen (O'Grady *et al.*, 2000). Sardines kept in MAP with 35-70% CO_2, 5% O_2 and 25-60% N_2 had the lower lipid oxidation and longer shelf-life, compared with those packed in air. The lowest lipid oxidation was found in MAP with 70% CO_2 (Erkan *et al.*, 2006). Fresh seabass (*Lates calcalifer*) slices stored under MAP (60-100% CO_2, 0-40% N_2 and 0-20% O_2) exhibited better odor, flavor and acceptability score in comparison with fish slices kept in air (Masniyom *et al.*, 2002). Brown *et al.* (1980) reported that silver salmon (*Oncorhynchus kisutch*) fillets stored in MAP (20-40% CO_2, 60-80% N_2 and 0-1% CO) could reduce the development of strong offensive aromas. Pantazi *et al.* (2008) also found that based on odor and taste attributes, fresh swordfish (*Xiphias gladius*) slices kept in air and MAP (40% CO_2, 30% N_2 and 30% O_2) had a shelf-life for 7 and 12 days, respectively.

MAP using a high CO_2 environment is an effective means of prolonging microbial shelf-life of meat during extended storage. Nevertheless, the use of high levels of CO_2, with a consequent low O_2 concentration, can cause meat discoloration (Fernandez-Lopez *et al.*, 2008). Discoloration of the characteristic bright red meat color is related to the conversion of oxyMb to metMb. This phenomenon may be counteracted by incorporation of CO to the atmosphere (Fernandez-Lopez *et al.*, 2008; Hunt *et al.*, 2004). Since 1985, the Norwegian meat industry has been using a gas mixture containing 0.3-0.5% CO (balance 60% CO_2 and 40% N_2) in retail-ready packages of beef, pork and lamb (Sorheim *et al.*, 1997). However, after 2 decades of

successful application, Norway discontinued the use of CO-MAP for red meat in July 2004 due to their entry into the European Union, which currently does not approve the use of CO in meat packaging (Wilkinson *et al.*, 2006). At the same time, CO-MAP became more relevant for the United States meat industry and was approved by the FDA for use at a level of 0.4% in MAP systems for red meat (Eilert, 2005). CO binds strongly to Mb to form a bright cherry red pigment, carboxyMb. From a biochemical aspect, it appears that deoxyMb is more readily converted to carboxyMb than is oxyMb or metMb. The absence of ligands in the sixth coordinate of heme iron in deoxyMb is widely considered as the most favorable reason for its reaction with CO (Mancini and Hunt, 2005). Lanier *et al.* (1978) observed that metMb reducing activity was enhanced in a CO-containing environment, possibly due to a stabilizing effect of CO on heme and by counteracting the inhibitory action of oxygen on enhanced reduction. Nevertheless, the reaction characteristics of CO with oxyMb and metMb and the interconversion between carboxyMb and other Mb forms are poorly understood.

Previous research revealed that CO-MAP decreased lipid oxidation and increased color stability in different meat systems (Chow *et al.*, 1997; Luno *et al.*, 2000; Sorheim *et al.*, 1999). Furthermore, Hunt *et al.* (2004) concluded that the use of 0.4% CO in MAP improved beef color without masking spoilage. After the removal of product from CO-MAP, meat color deteriorated during display in a manner similar to that of oxyMb. The absorption spectrum of carboxyMb is very similar to that of oxyMb (Suman *et al.*, 2006). For carboxyMb spectra, the magnitude of the absorbance values at 543 nm was consistently greater than at 581 nm, whereas in oxyMb the reverse was noticeable. Traditional equations used to estimate the relative proportions of Mb redox forms in aqueous solutions/meat extracts have not included carboxyMb and are based on the wavelength maxima for oxyMb, deoxyMb and metMb (Krzywicki, 1982; Tang *et al.*, 2004). Therefore, the application of these equations to carboxyMb containing solutions would be inappropriate, because they do not account for the wavelength maxima and extinction coefficient of carboxyMb. Furthermore, the nearly identical spectra of carboxyMb and oxyMb make it very difficult to determine the relative proportions of these two species (Suman *et al.*, 2006).

1.3 Objectives

1. To monitor the changes of Mb and lipids as well as the development of fishy and rancid off-odor in seabass and red tilapia muscles during iced storage
2. To purify and characterize Mb from Eastern little tuna
3. To investigate the different factors on the changes in Eastern little tuna Mb
4. To study the impact of fish Mb on lipid oxidation in washed fish mince at varying pH
5. To compare the impact of fish Mb and Hb with different forms on lipid oxidation in washed fish mince
6. To investigate the inhibitory effect of different phenolic compounds on lipid oxidation in washed fish mince induced by Mb and Hb
7. To elucidate the inhibitory effect of phenolic compounds in combination with high CO_2-modified atmosphere packaging on lipid oxidation and quality changes of Eastern little tuna slices during refrigerated storage

CHAPTER 2

Changes in heme proteins and lipids associated with off-odor of seabass (*Lates calcarifer*) and red tilapia (*Oreochromis mossambicus* × *O. niloticus*) during iced storage

2.1 Abstract

Changes in myoglobin (Mb) and lipid associated with off-odor development in seabass (*Lates calcarifer*) and red tilapia (*Oreochromis mossambicus* × *O. niloticus*) muscles during 15 days of iced storage were studied. Fresh seabass contained the higher contents of Mb and heme iron, compared with red tilapia ($P < 0.05$). An increase in metMb proportion was observed during storage. After 3 days of storage, a decreased heme iron content and a concomitant increased non-heme iron content were noticeable in both fish ($P < 0.05$). Oxidation of Mb and released non-heme iron were associated with lipid oxidation. Decreases in unsaturated fatty acids, especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), along with an increase in oxidation products and free fatty acids were observed as the storage time progressed. Fishy and rancid odors were detected at day 6 of storage for both fish and higher intensity was found in seabass muscle. Thus, the off-odor in fish muscle was mostly governed by lipid oxidation and species specific.

2.2 Introduction

Odor and color of raw fish are the important quality attributes for purchasing decisions of consumers. The loss in quality is more pronounced with the extended post-mortem storage, especially under the inappropriate condition. Lipid deterioration is one of important reactions, which limits the shelf-life of fresh fish (Chaijan *et al.*, 2006; Sohn *et al.*, 2005). The lipids of post-mortem fish muscle are prone to oxidation due to the higher content of unsaturated fatty acids than those of mammals and birds (Nawar, 1996; Sohn *et al.*, 2005). Lipid oxidation is

a chain reaction consisting of initiation, propagation and termination reactions. This phenomenon can be influenced by both intrinsic and extrinsic factors such as the fatty acid composition, the concentration of pro-oxidants, endogenous ferrous iron, heme protein, enzymes, pH, temperature, ionic strength and oxygen consumption (Chan *et al.*, 1997; Grunwald and Richards, 2006; Nawar, 1996; Sohn *et al.*, 2005).

Heme proteins hemoglobin (Hb) and myoglobin (Mb), likely present in the post-mortem muscle have a close relationship with lipid oxidation. Chan *et al.* (1997) reported a pro-oxidative effect of oxyMb in liposome systems. The lipid oxidation in fish muscle was promoted by the oxidation of Mb, which played a considerable role in the generation of hydroperoxides (Sohn *et al.*, 2005). Hydroperoxides are readily decomposed to a variety of volatile compounds, including aldehydes, ketones and alcohols, which mainly contribute to the development of undesirable odors in fish flesh. Higher contents of polyunsaturated fatty acid and Mb found in yellowtail dark muscle are the important factors associated with the susceptibility to lipid oxidation and generation of rancid off-odor under refrigerated conditions (Sohn *et al.*, 2005). However, the development of fishy and rancid off-odor can be found in a lean fish with increasing storage time (Masniyom *et al.*, 2002).

Seabass (*Lates calcarifer*) and red tilapia (*Oreochromis mossambicus* × *O. niloticus*) are very popular in South-East Asia owing to their white flesh and delicacy. In general, they are sold as whole fish or as fillets. Iced storage has been widely used to prolong their shelf-life, particularly during transportation and distribution. However, those fish express fishy or off-odor, which lowers the market value and consumer acceptance. Such off-odor may be associated with the changes in heme pigments and lipids localized in fish muscle. However, a limited work regarding the oxidation of fish Mb and lipids and their relation with fishy odor in fish muscle has been carried out. Thus, the objective of this study was to investigate the changes of Mb and lipids as well as the development of fishy and rancid off-odor in seabass and red tilapia muscles during iced storage.

2.3 Materials and Methods

2.3.1 Chemicals

Palmitic acid, cupric acetate, pyridine and bathophenanthroline disulfonic acid were purchased from Sigma (St. Louis. MO, USA). Trichloroacetic acid, anhydrous sodium sulfate, sodium nitrite, isooctane, ferrous chloride and iron standard solution were obtained from Merck (Darmstadt, Germany). Disodium hydrogen phosphate, sodium dihydrogen phosphate, 2-thiobarbituric acid and 1,1,3,3-tetramethoxypropane were procured from Fluka (Buchs, Switzerland). Methanol, acetone, chloroform and ammonium thiocyanate were obtained from Lab-Scan (Bangkok, Thailand).

2.3.2 Fish samples

Seabass (*Lates calcarifer*) with the average weight of 0.5-0.55 kg was purchased from a brackish-water farm in Koyo Island, Songkhla province, Thailand. Hybrid red tilapia (*Oreochromis mossambicus* × *O. niloticus*) having the average weight of 0.57-0.62 kg was caught from a fresh-water farm in Phattalung province, Thailand. After capture, fish were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 2 h. Whole fish without evisceration were immediately washed and kept in ice with a fish/ice ratio of 1:2 (w/w). The fish were placed and distributed uniformly between the layers of ice and kept for 15 days at room temperature (28-30ºC). To maintain the fish/ice ratio, molten ice was removed and replaced every 2 days. During storage, eight fish was randomly taken as the composite sample at day 0, 3, 6, 9, 12 and 15 for analyzes. The fish samples were washed and filleted. The flesh was chopped to uniformity and used for analyzes. Fat contents of fresh seabass and red tilapia muscle were determined (AOAC, 2000) to be 3.03 and 1.83%, respectively. Three different lots of fish were used for the whole study.

2.3.3 Measurement of Mb content

Mb content of dorsal muscle from seabass and red tilapia was determined by spectrophotometric method as described by Benjakul and Bauer (2001). Ground sample (2 g) was weighed and transferred into a 50-ml polypropylene centrifuge tube and 20 ml of cold 40 mM

phosphate buffer (pH 6.8) were added. The mixture was homogenized at 13,500 rpm using an IKA Labortechnik homogenizer (Selangor, Malaysia) for 10 sec, followed by centrifuging at 3000*g* for 30 min at 4ºC using an Avanti J-E centrifuge (Beckman Coulter, Palo Alto, CA, USA). The supernatant was filtered with Whatman No. 1 filter paper (Schleicher & Schuell, Maidstone, England). The absorbance was read at 525, 545, 565 and 572 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Mb content was calculated using the molar extinction coefficient of 7.6×10^{-3} and a molecular weight of 16,110 (Gomez-Basauri and Regenstein, 1992; Krzywicki, 1982). The proportions of different Mb forms including deoxyMb, oxyMb and metMb were calculated by Krzywicki's equations (Krzywicki, 1982).

2.3.4 Measurement of heme and non-heme iron contents

Heme iron content of fish muscle was determined as described by Lee *et al.* (1999) and Cheng and Ockerman (2004) with a slight modification. Ground sample (2 g) was mixed with 9 ml of acid acetone (90% acetone, 8% deionized water and 2% HCl, v/v/w). The mixture was macerated with a glass rod and allowed to stand for 1 h at room temperature. The extract was filtered with a Whatman No. 42 filter paper and the absorbance was read at 640 nm against an acid acetone used as blank. Heme iron content was calculated with the factor of 0.0882 µg/µg hematin using the following formula:

$$\text{Heme iron (ppm)} = \text{Total pigment (ppm)} \times 0.0882$$

where total pigment (ppm) = $A_{640} \times 680$. The heme iron content was expressed as µmol/100 g sample.

Non-heme iron content of fish muscle was determined according to the method of Schricker *et al.* (1982). Ground sample (1.0 g) was weighed and transferred into a screw cap test tube and 50 µl of 0.39% (w/v) sodium nitrite were added. Four ml of a mixture of 40% trichloroacetic acid and 6 M HCl (ratio of 1: 1 (v/v), prepared freshly) were added. The tightly capped tubes were placed in an incubator shaker (W350, Memmert, Schwabach, Germany) at 65ºC for 22 h and then cooled at room temperature for 2 h. The supernatant (400 µl) was mixed with 2 ml of the non-heme iron color reagent, a mixture of bathophenanthroline disulfonate reagent (0.162 g of bathophenanthroline disulfonic acid + 100 ml of double-deionized water + 2

ml of thioglycolic acid), double-deionized water and saturated sodium acetate solution at a ratio of 1:20:20 (v/v/v), prepared freshly. After vortexing and standing for 10 min, the absorbance was measured at 540 nm. The non-heme iron content was calculated from iron standard curve. The iron standard solutions ($Fe(NO_3)$ in HNO_3), with the concentrations ranging from 0 to 2 ppm, were used.

2.3.5 Lipid extraction

Lipid was extracted by the Bligh and Dyer method (Bligh and Dyer, 1959). Sample (25 g) was homogenized with 200 ml of a chloroform:methanol:distilled water mixture (50:100:50, v/v/v) at the speed of 9,500 rpm for 2 min at 4ºC. The homogenate was added with 50 ml of chloroform and homogenized at 9,500 rpm for 1 min. Thereafter, 25 ml of distilled water were added and homogenized at the same speed for 30 sec. The homogenate was centrifuged at 3000*g* at 4ºC for 15 min and transferred into a separating flask. The chloroform phase was drained off into the 125 ml Erlenmeyer flask containing about 2-5 g of anhydrous sodium sulphate, shaken very well and decanted into a round-bottom flask through a Whatman No.4 filter paper. The solvent was evaporated at 25ºC using an EYELA rotary evaporator N-1000 (Tokyo, Japan) and the residual solvent was removed by flushing nitrogen.

2.3.6 Measurement of conjugated diene (CD)

Conjugated diene was measured according to the method of Frankel *et al.* (1996). Oil sample (0.1 g) was dissolved in 5.0 ml of isooctane and the absorbance was measured at 234 nm.

2.3.7 Measurement of peroxide value (PV)

Peroxide value was determined using the ferric thiocyanate method with a slight modification (Chen *et al.*, 1995; Mitsuda *et al.*, 1966). To 50 µl of diluted oil sample (10-fold dilution), 2.35 ml of 75% ethanol (v/v), 50 µl of 30% ammonium thiocyanate (w/v) and 50 µl of 20 mM ferrous chloride solution in 3.5% HCl (w/v) were added and mixed thoroughly. After 3 min, the absorbance of the colored solution was read at 500 nm. Blank was prepared in the same

manner, except the distilled water was used instead of ferrous chloride. PV was expressed as A_{500} after blank substraction.

2.3.8 Measurement of thiobarbituric acid-reactive substances (TBARS)

Thiobarbituric acid-reactive substances (TBARS) were determined as described by Buege and Aust (1978). Ground sample (0.5 g) was homogenized with 2.5 ml of a solution containing 0.375% thiobarbituric acid (w/v), 15% trichloroacetic acid (w/v) and 0.25 M HCl. The mixture was heated in a boiling water bath (95-100ºC) for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3600*g* at 25ºC 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 the concentrations ranging from 0 to 6 ppm. TBARS was calculated and expressed as mg malonaldehyde/kg sample.

2.3.9 Measurement of free fatty acid (FFA)

Free fatty acid content was determined according to the method of Lowry and Tinsley (1976). Lipid sample (0.1 g) was added with 5 ml of isooctane and swirled vigorously to dissolve the sample. The mixture was then treated with 1 ml of 5% (w/v) cupric acetate-pyridine reagent, prepared by dissolving 5 g of the reagent grade cupric acetate in 100 ml of water, filtering and adjusting the pH to 6.0-6.2 using pyridine. The mixture was shaken vigorously for 90 sec using a Vortex-Genie2 mixer (Bohemia, NY, USA) and allowed to stand for 10-20 sec. The upper layer was subjected to absorbance measurement at 715 nm. A standard curve was prepared using palmitic acid in isooctane at concentrations ranging from 0 to 50 μmol/5 ml. FFA content was expressed as g FFA/100 g lipid.

2.3.10 Determination of fatty acid profile

Fatty acid profile was determined as fatty acid methyl esters (FAMEs). The FAMEs were prepared according to the method of AOAC (2000). The prepared methyl ester was injected to the gas chromatography (Shimadzu, Kyoto, Japan) equipped with the flame ionization detector (FID) at a split ratio of 1:20. A fused silica capillary column (30 m × 0.25 mm), coated with bonded polyglycol liquid phase, was used. The analytical conditions were: injection port

temperature of 250ºC and detector temperature of 270ºC. The oven was programmed from 170 to 225ºC at a rate of 1ºC/min (no initial or final hold). Retention times of FAME standards were used to identify chromatographic peaks of the samples. Fatty acid content was calculated, based on the peak area ratio and expressed as g fatty acid/100 g oil.

2.3.11 Fourier transform infrared (FTIR) spectra analysis

FTIR analysis of crude oil was performed in a horizontal ATR Trough plate crystal cell (45º ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology, Inc., Madison, WI, USA) equipped with a Bruker Model Vector 33 FTIR spectrometer (Bruker Co., Ettlingen, Germany). Prior to analysis, the crystal cell was cleaned with acetone, wiped dry with soft tissue and the background scan was run. For spectra analysis, oil sample (200 µl) was applied directly onto the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectra in the range of 4000-400 cm^{-1} (mid-IR region) with the automatic signal gain were collected in 16 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean, empty cell at 25ºC. Analysis of spectral data was carried out using the OPUS 3.0 data collection software program (Bruker Co., Ettlingen, Germany).

2.3.12 Measurement of trimethylamine (TMA)

TMA contents were determined according to the method of Conway and Byrne (1933). Ground sample (5 g) was added with 20 ml of 4% trichloroacetic acid (w/v) and homogenized at a speed of 11,000 rpm for 1 min. The homogenate was filtered using Whatman No. 4 filter paper and the filtrate was used for analysis. In the outer ring, formaldehyde (10%, w/v) (1 ml) was added to the filtrate (1 ml) to fix ammonia present in sample. To initiate the reaction, saturated K_2CO_3 (1 ml) was mixed with the prepared sample to release TMA. TMA was trapped in 1 ml of the inner ring solution (1% boric acid (w/v) containing the Conway indicator). The Conway unit was incubated at 37ºC for 60 min. The titration of inner ring solution was performed using 0.02 M HCl and the amount of TMA was calculated. TMA content was expressed as mg N/100 g sample.

2.3.13 Sensory evaluation

Sensory evaluation was performed by 6 trained panelists who had the extensive experience in the evaluation of off-odors and off-flavors of raw fish muscles. The intensities of fishy, rancid and spoiled odor as well as overall off-odor were evaluated using a 5-point scale ranging from none (score=0) to strong (score=4) (Sohn *et al.*, 2005).

2.3.14 Statistical analysis

Experiments were run in triplicate using three lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

2.4 Results and Discussion

2.4.1 Changes in Mb of seabass and red tilapia muscle during iced storage

Mb contents in fresh seabass and red tilapia muscle were 2.61 and 1.51 mg/g sample, respectively. In general, Mb content is highly related to the metabolic pattern and types of muscle (Chaijan *et al.*, 2005). The changes in proportion of different Mb forms of seabass and red tilapia muscle during iced storage are shown in Table 3. Muscle of both fish possessed deoxyMb as the predominant form, followed by metMb and oxyMb, respectively. Physiologically active forms of Mb are iron (II) Mbs involving deoxy- and oxyMb content. Increase in metMb content was found in seabass and red tilapia muscle after day 3 and 9 of iced storage, respectively ($P < 0.05$) and related with a decrease in redness index of both fish muscles (data not shown). Normally, the increase in metMb formation can be observed during iced storage of fish muscle (Chaijan *et al.*, 2005; Sohn *et al.*, 2005). Increased metMb proportion in fish sample indicated that ferrous Mb underwent the oxidation to form ferric metMb during the extended storage. The constant metMb proportion at the early stage of storage for both fish species was probably due to the result of metMb reductase. It has been known that metMb reductase is a component of red blood cells and can be found in fish muscle (Al-Shaibani *et al.*, 1977). MetMb reductase remaining in the muscle might reduce metMb to other forms. The inactivation of metMb

reductase was also presumed to be associated with the increased formation of metMb. For deoxyMb, no changes were noticeable in red tilapia during storage, while a decrease was found in seabass only within the first 3 days of storage ($P < 0.05$). A continuous oxidation to metMb takes place in biological tissue, though the subsequent enzymatic reduction to the deoxy-forms occurs (Al-Shaibani *et al.*, 1977). OxyMb content tended to decrease with increasing storage time and the lowered oxyMb content was obtained in seabass and red tilapia muscle after day 12 and 9, respectively ($P < 0.05$). The lowest oxyMb content with the concomitant highest metMb content was found at day 15 of storage ($P < 0.05$). Therefore, Mb in the muscle of both species most likely underwent oxidation, in which metMb was formed with the decrease in oxyMb content during the extended storage. During 15 days of storage, pH increased from 6.3 to 6.7 for seabass muscle and from 6.5 to 6.9 for red tilapia muscle.

Table 3. Proportion of different Mb forms (%) in seabass and red tilapia ordinary muscles during 15 days of iced storage*

Storage time (days)	Seabass			Red tilapia		
	%deoxyMb	%oxyMb	%metMb	%deoxyMb	%oxyMb	%metMb
0	40.36 ± 0.42^{b}	8.70 ± 1.09^{bc}	37.10 ± 0.75^{a}	40.23 ± 0.73^{a}	10.75 ± 0.82^{bc}	33.32 ± 0.91^{a}
3	39.58 ± 0.58^{a}	8.89 ± 0.74^{c}	37.83 ± 0.84^{a}	41.86 ± 1.16^{a}	10.95 ± 0.59^{bc}	32.05 ± 1.76^{a}
6	39.71 ± 0.14^{ab}	7.76 ± 0.15^{b}	39.13 ± 0.12^{b}	41.18 ± 0.60^{a}	12.03 ± 0.79^{c}	32.07 ± 0.17^{a}
9	39.52 ± 0.49^{a}	8.11 ± 0.15^{bc}	39.21 ± 0.69^{b}	42.05 ± 0.37^{a}	9.79 ± 0.83^{b}	33.77 ± 1.38^{a}
12	39.58 ± 0.04^{a}	7.94 ± 0.00^{bc}	39.21 ± 0.05^{b}	42.10 ± 2.43^{a}	6.66 ± 0.68^{a}	37.70 ± 2.69^{b}
15	39.35 ± 0.24^{a}	5.99 ± 0.32^{a}	42.34 ± 0.41^{c}	41.82 ± 0.11^{a}	6.49 ± 0.56^{a}	38.52 ± 0.91^{b}

***** Different letters in the same column indicate significant differences ($P < 0.05$)

****** pH of buffer used for measurement was 6.8

2.4.2 Changes in heme iron and non-heme iron contents of seabass and red tilapia muscle during iced storage

The changes of heme iron content in seabass and red tilapia muscle during iced storage are depicted in Figure 7A. Fresh seabass muscle had a higher heme iron content (12.31

μmol/100 g) than did red tilapia muscle (10.06 μmol/100 g). The presence of larger amounts of heme iron in seabass muscle correlated well with the higher Mb content. Hb, Mb as well as mitochondrial iron-containing enzymes are the sources of iron in fish muscle (Decker and Hultin, 1990; Hazell, 1982). Decreases in heme iron contents were observed in muscles of both fish up to day 9 of storage ($P < 0.05$). Thereafter, an increase was found in seabass muscle ($P < 0.05$), while a negligible change was noticeable in red tilapia muscle ($P > 0.05$). At day 15, heme iron content of seabass and red tilapia muscles decreased by 45% and 27%, respectively, compared with that obtained in fresh muscle. Benjakul and Bauer (2001) reported that heme iron content in 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. Heme iron content most likely correlated with total pigment content (Chaijan *et al.*, 2005; Cheng and Ockerman, 2004; Lee *et al.*, 1999). Slight increase in heme iron content found at the end of storage of seabass muscle was possibly associated with the increased degradation of fish muscle, leading to the ease of heme extraction. Decker and Hultin (1990) reported that the deterioration of subcellular organelles, e.g. mitochondria, and the release cytochorme c, could be responsible for the increase in soluble hemin. The higher rate of decrease in heme iron content observed in seabass muscle with extended storage time suggested that the higher disruption of heme proteins and higher release of iron from heme occurred in seabass muscle than in red tilapia muscle.

Non-heme iron contents in seabass and red tilapia muscles during iced storage are depicted in Figure 7B. For fresh muscle, similar non-heme iron content was obtained between both seabass and red tilapia ($P > 0.05$). For seabass, the increase in non-heme iron content was found within the first 6 days of storage ($P < 0.05$). Thereafter, no changes were observed up to 15 days of storage ($P > 0.05$). However, a continuous increase in non-heme iron content was noticeable in red tilapia throughout the storage of 15 days ($P < 0.05$). In general, the higher content of non-heme iron was found in seabass muscle, in comparison with red tilapia muscle, particularly during 6-9 days of storage ($P < 0.05$). Schricker *et al.* (1982) and Chaijan *et al.* (2005) reported that non-heme iron concentrations were significantly different between species and muscle types. The increase in non-heme iron content was in agreement with the decrease in heme iron content. The higher Mb and heme iron content of fresh seabass were related with the higher non-heme iron content of muscle during the extended storage. Benjakul and Bauer (2001)

and Chaijan *et al.* (2005) reported the increased non-heme iron in catfish, sardine and mackerel with extended iced storage time. These results suggested that the disruption of porphyrin ring probably occurred during storage, leading to the release of free iron named "non-heme iron". Therefore, non-heme iron content in muscle of both fish could be released to a greater extent during iced storage. This free iron might act as a pro-oxidant in fish muscle and was associated with the enhanced lipid oxidation.

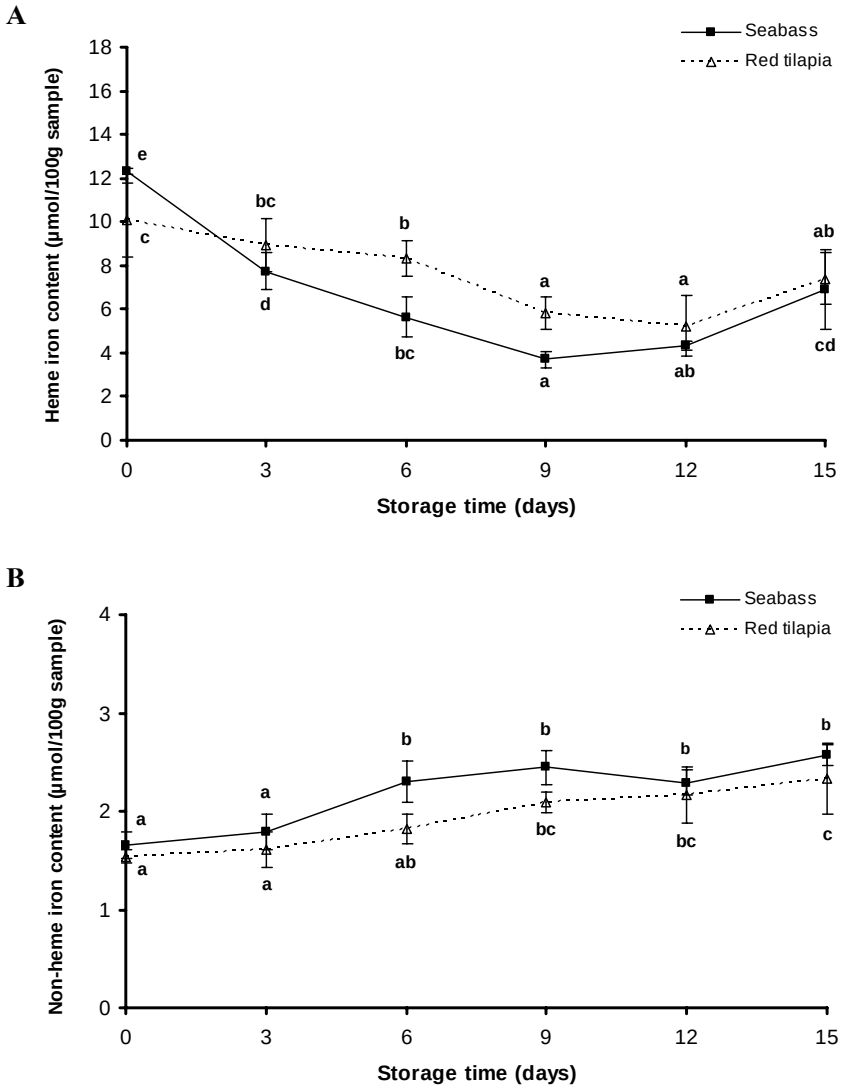


Figure 7. Changes in heme iron content (A) and non-heme iron content (B) of seabass and red tilapia muscles during iced storage. Different letters within the same fish muscle indicate significant differences ($P < 0.05$). Bars represent the standard deviation (n=3)

2.4.3 Lipid oxidation of seabass and red tilapia muscle during iced storage

Lipid oxidation in seabass and red tilapia muscle during iced storage was measured by monitoring CD, PV and TBARS values (Figure 8). Generally, lipid oxidation occurs in fish muscle during the extended storage (Chaijan *et al.*, 2006). Initiation reactions take place either by the abstraction of hydrogen radical 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 (CH_2=CH-CH=CH_2) (Frankel *et al.*, 1996). As shown in Figure 8A, the increase in CD was observed in seabass muscle up to 9 days of iced storage and the constant CD value was obtained thereafter ($P > 0.05$). No changes in CD were found in red tilapia muscle within the first 12 days of storage ($P > 0.05$) but an increase in CD was noticeable at day 15 ($P < 0.05$). The differences in CD between both fish indicated the different rate of oxidation at the initial stage, where the abstraction of hydrogen atom was more pronounced in seabass muscle. After 15 days of iced storage, lipid oxidation was enhanced as shown by a higher CD formation ($P < 0.05$).

The increase in PV was noticeable in both fish within the first 3 days of iced storage ($P < 0.05$) (Figure 8B). After 3 days of iced storage, a gradual decrease in PV was found in red tilapia muscle ($P < 0.05$), whereas there was no change in PV in seabass muscle up to 15 days of storage ($P > 0.05$). Slight decreases in PV during storage suggested that hydroperoxide formed might be decomposed to other compounds. Lipid hydroperoxides are formed by various pathways including the reaction of singlet oxygen with unsaturated lipids or the lipoxygenase-catalyzed oxidation of polyunsaturated fatty acids (Nawar, 1996). A continuous increase in TBARS in red tilapia muscle was found up to 15 days of storage ($P < 0.05$) (Figure 8C). For seabass muscle, the increase in TBARS was found up to 9 days of storage, followed by the decrease until the end of storage (day 15) ($P < 0.05$). The decrease in TBARS might be caused by a loss of low molecular weight decomposition products during the advancement of oxidation (Nawar, 1996). Adduction of aldehyde with other compounds, especially proteins, might contribute to the lowered TBARS.

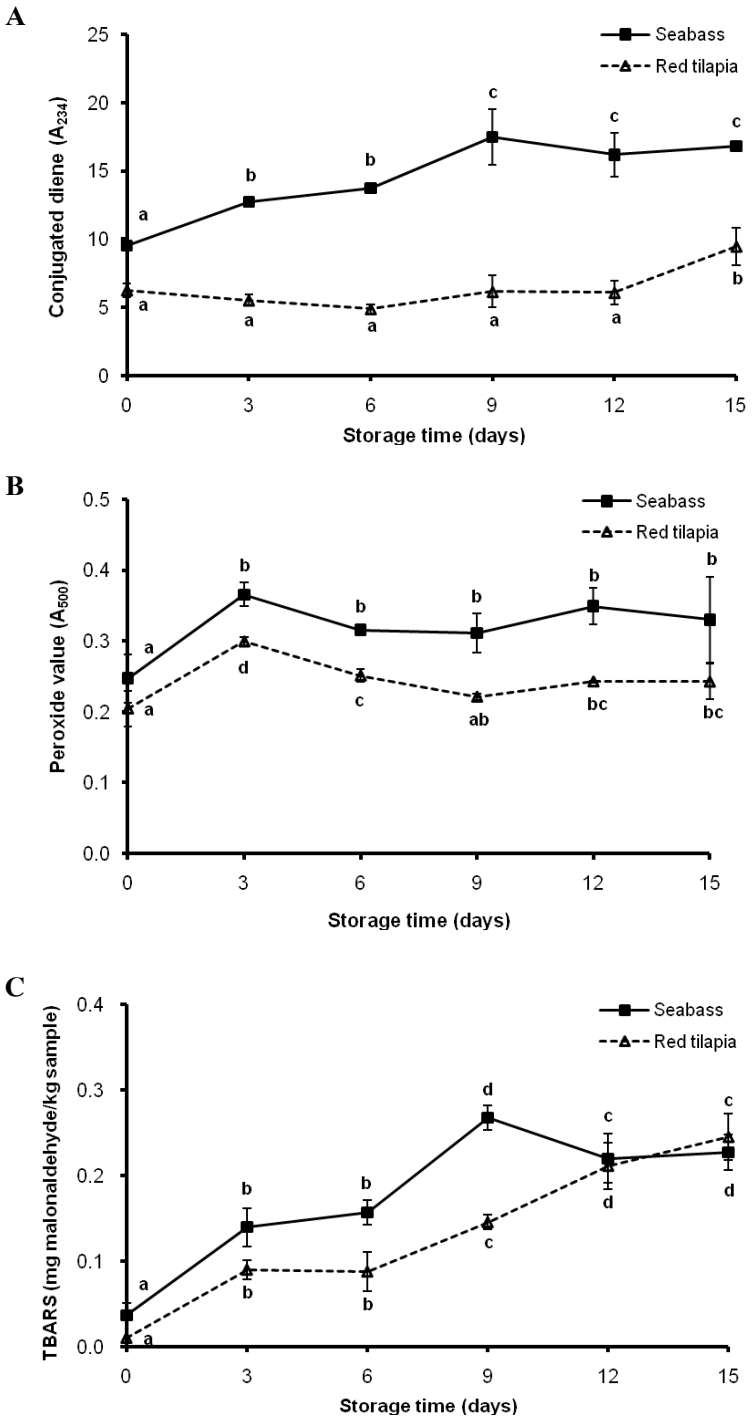


Figure 8. Changes in conjugated diene (A), peroxide value (B) and TBARS value (C) of seabass and red tilapia muscles during iced storage. Different letters within the same fish muscle indicate significant differences ($P < 0.05$). Bars represent the standard deviation (n=3)

Lipid oxidation in fish muscle could be governed by the contents and forms of Mb as well as non-heme iron (Grunwald and Richards, 2006; Lee *et al.*, 2003; Sohn *et al.*, 2005). From the result, a decrease in oxyMb proportion as well as an increase in metMb proportion during iced storage was coincidental with an increase in lipid oxidation of both muscles. Thus, autoxidation of Mb could be associated with enhanced lipid oxidation. Autoxidation of oxyMb results in the formation of metmyogobin and superoxide, which rapidly dismutate to H_2O_2 and oxygen. The interaction of H_2O_2 with metMb led very rapidly to generation of an active species, ferryl radical, which could initiate lipid peroxidation (Baron *et al.*, 2002). During iced storage of both fish, the increases in TBARS formation coincided with the formation of metMb, which might accelerate lipid oxidation in fish muscle. Apart from Mb, Hb in fish muscle was able to be an effective catalyst of lipid oxidation (Apte and Morrissey, 1987). Heme dissociation, heme destruction and iron release play a role in the acceleration of lipid oxidation (Grunwald and Richards, 2006).

2.4.4 Lipid hydrolysis of seabass and red tilapia muscle during iced storage

Lipid hydrolysis occurred in seabass and red tilapia muscle during iced storage (Figure 9). FFA content in seabass muscle increased as storage time increased ($P < 0.05$). For red tilapia, no change in FFA content was observed within the first 6 days ($P > 0.05$) but a marked increase was noticeable during 6 and 15 days of iced storage ($P < 0.05$). At the end of storage period, lipid hydrolysis occurred to a great extent as evidenced by the highest FFA content obtained in muscle of both fish. Lipases, phospholipase A and phospholipase B are important enzymes involved in hydrolysis of fish lipids (Hwang and Regenstein, 1993). FFA can undergo further oxidation to produce low molecular weight compounds that are responsible for the rancidity of fish and fish products (Nawar, 1996). Fish muscle containing high free fatty acid was more prone to lipid oxidation. In the presence of non-heme iron as well as metMb, the increased oxidation of free fatty acids, particularly polyunsaturated fatty acid, was obtained as indicated by the increases in TBARS during the extended storage (Figure 8C). Baron *et al.* (2002) reported a concentration effect of free fatty acid anion linoleate on pro-oxidative activity of metMb in emulsion system. At low linoleate/heme protein ratio, metMb acted as the ineffective initiators of lipid oxidation, whereas, at higher ratio, metMb possessed a strong pro-oxidative activity.

Therefore, a high content of FFA might result in the advanced lipid oxidation initiated by metMb, especially with increasing storage time.

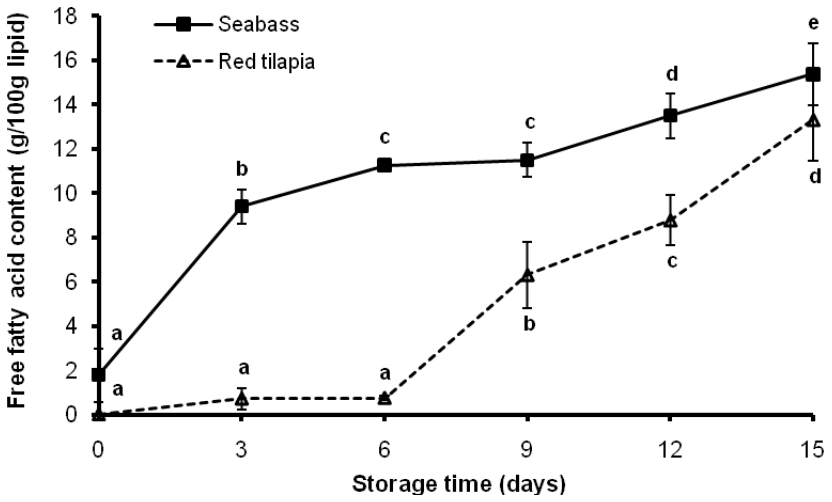


Figure 9. Changes in free fatty acid content of seabass and red tilapia muscles during iced storage. Different letters within the same fish muscle indicate significant differences ($P < 0.05$). Bars represent the standard deviation (n=3)

2.4.5 Fatty acid profiles of fresh and ice-stored seabass and red tilapia muscle

Fatty acid profiles of seabass and red tilapia muscle at day 0 and day 15 of iced storage are shown in Table 4. Lipid of fresh seabass muscle contained 40.32% SAT, 17.18% MUFA and 35.77% PUFA, whereas lipid of fresh red tilapia lipid muscle consisted of 34.65% SAT, 40.87% MUFA and 21.46% PUFA. Seabass muscle contained palmitic acid (C16:0) as the most abundant fatty acid, followed by docosahexaenoic acid (C22:6(*n*-3)). For red tilapia, oleic acid (C18:1(*n*-9)) was the dominant fatty acid, followed by palmitic acid (C16:0) and linoleic acid (C18:2(*n*-6)). PUFAs were found to be different between seabass and red tilapia muscle. For seabass muscle, the contents of DHA and EPA were 19.21 and 5.27 g/100 g oil, respectively, whereas DHA and EPA at levels of 3.14 and 0.13 g/100 g oil, respectively, were found in red tilapia muscle. These fatty acids are beneficial to the human health, being essential for the development and functionality of certain organs and several physiological and biochemical responses of organisms (Nawar, 1996). After 15 days of iced storage, DHA and EPA decreased

Table 4. Fatty acid profiles of fresh and 15 days-ice-stored seabass and red tilapia muscle

Fatty acids (g/100 g oil)	Seabass		Red tilapia	
	Day 0	Day 15	Day 0	Day 15
C12:0	0.12	0.08	0.08	0.08
C14:0	4.23	4.35	2.98	2.92
C14:1	0.14	0.15	0.15	0.18
C15:0	0.91	0.94	0.17	0.18
C16:0	23.85	24.73	24.40	24.23
C16:1 *n*-7	4.99	5.25	4.82	5.59
C17:0	1.57	1.62	0.25	0.24
C18:0	8.76	9.30	6.35	5.07
C18:1 *n*-9	9.10	9.73	34.27	35.01
C18:1 *n*-7	2.58	2.62	ND	ND
C18:2 *n*-6	1.09	1.01	13.19	13.97
C18:3 *n*-3	0.50	0.55	0.68	0.65
C18:3 *n*-6	0.16	0.16	0.52	0.53
C18:4 *n*-3	0.41	0.44	0.07	0.06
C20:0	0.57	0.63	0.20	0.18
C20:1 *n*-9	0.23	0.24	1.45	1.34
C20:2 *n*-6	0.18	0.17	0.79	0.80
C20:3 *n*-6	0.15	0.16	0.67	0.59
C20:3 *n*-3	0.09	0.11	0.14	0.13
C20:4 *n*-6	3.96	3.33	0.81	0.86
C20:4 *n*-3	0.29	0.30	0.08	0.06
C20:5 *n*-3 (EPA)	5.27	4.95	0.13	0.09
C21:0	ND	0.18	0.08	ND
C22:0	ND	ND	ND	0.06
C22:1 *n*-9	0.07	0.08	0.10	0.06
C22:1 *n*-11, *n*-13	0.07	0.07	0.07	0.06
C22:4 *n*-6	0.21	0.19	0.06	0.05
C22:5 *n*-6	1.92	1.74	0.49	0.52
C22:5 *n*-3	2.33	2.35	0.67	0.51
C22:6 *n*-3 (DHA)	19.21	18.10	3.14	2.78
C23:0	0.09	0.07	0.08	0.10
C24:0	0.22	0.17	0.04	ND
Saturated fatty acid (SFA)	**40.32**	**42.07**	**34.65**	**33.06**
Monounsaturated fatty acid (MUFA)	**17.18**	**18.16**	**40.87**	**42.25**
Polyunsaturated fatty acid (PUFA)	**35.77**	**33.55**	**21.46**	**21.61**

ND: non-detectable

by 5.78 and 6.04%, respectively, for seabass lipid and by 11.63 and 33.13%, respectively, for red tilapia lipid. The decreases in DHA and EPA contents suggested their susceptibility to oxidation during the extended storage. During iced storage, triglycerides and phospholipids underwent hydrolysis into free fatty acids (Figure 9), which were prone to oxidation (Figure 8). It was noted that the proportion of PUFA in seabass muscle decreased with the coincidental increase in SFA and MUFA contents after 15 days of iced storage. Nevertheless, no marked change in PUFA content was found in red tilapia muscle.

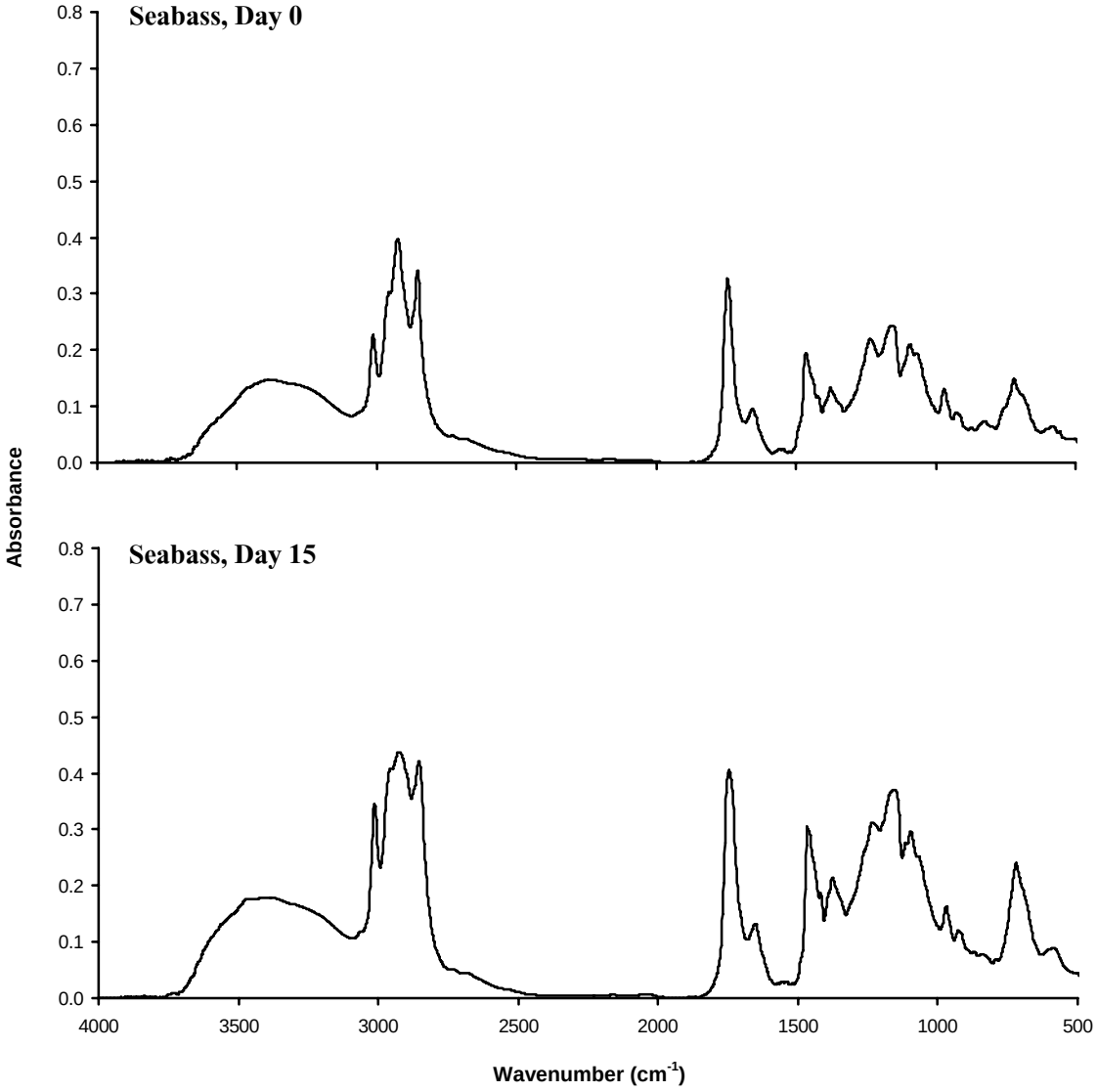
2.4.6 FTIR spectra of lipid from fresh and ice-stored seabass and red tilapia muscle

FTIR spectra of lipid extracted from seabass and red tilapia muscle stored in ice at day 0 and day 15 are shown in Figure 10. Dominant peaks were found at wavenumber range of 3050-2800 cm^{-1}, representing CH stretching vibrations, which overlap with —OH group in carboxylic acids (3100-2400 cm^{-1}). Higher amplitude of peak with wavenumber of 3600-3100 cm^{-1} representing —OH, —NH, ≡CH and =C—H stretching was observed in seabass muscle, compared with red tilapia muscle, regardless of storage time. This was possibly due to the higher amounts of diacylglycerol and unsaturated hydrocarbons in seabass muscle, reflecting by the higher FFA content found in this species (Figure 10). Hydroperoxide moieties exhibit the characteristic absorption peak between 3600 and 3400 cm^{-1} due to their —OO—H stretching vibrations (Van de Voort *et al.*, 1994). A larger peak representing hydroperoxide was found in seabass samples than that of red tilapia samples. It was noted that the higher amplitude was found in lipid from seabass stored in ice for 15 days, compared with that from fresh sample. On the other hand, the lower amplitude was observed in lipid extracted from red tilapia muscle stored in ice for 15 days. This was probably due to the loss or decomposition of hydroperoxides formed in red tilapia muscle to yield the secondary lipid oxidation products. Guillen and Cabo (2004) reported that the ratio between the absorbance band at 2854 cm^{-1}, due to the symmetrical stretching vibration of $—CH_2$ groups and the absorbance band between 3600 and 3100 cm^{-1} ($A_{2854}/A_{3600\text{-}3100}$) could be used to point out the changes in oxidation process. When a ratio of $A_{2854}/A_{3600\text{-}3100}$ was determined for seabass lipid, a slight change was found after 15 days of storage. This could be explained by the lower initial rate of oxidation process at the end of storage. Nevertheless, a higher ratio of $A_{2854}/A_{3600\text{-}3100}$ was observed in red tilapia sample after 15

days of storage, indicating the advanced oxidation process. This was concomitant with a rapid increase in TBARS of red tilapia at day 15 of storage (Figure 8). After 15 days of iced storage, the higher amplitude of peak at the wavenumber of 2850 and 2750 cm^{-1} was observed in both samples. The increased amplitude in this region indicated the higher amounts of aldehyde formed in both fish species at the end of storage.

Generally, the ester carbonyl functional group of triglycerides was observed at the wavenumber of 1741-1746 cm^{-1} (Setiowaty *et al.*, 2000). Peaks at wavenumbers of 1744 and 1745 cm^{-1} were considered to be a triglyceride peak for seabass and red tilapia muscle, respectively. After 15 days of storage, a shift towards lower wavenumber was found in both samples. These changes were associated with an increase in peak amplitude with a wavenumber of 1711 cm^{-1}, which represents the C=O carboxylic group of free fatty acids (Guillen and Cabo, 1997). The increases in free fatty acids as indicated by the increase in peak of 1711 cm^{-1} were in accordance with the increase in FFA for both fish muscles as shown in Figure 9. The peaks found at 1163 and 1237 cm^{-1} were associated with the stretching vibration of the C—O ester groups and the bending vibration of CH_2 groups, and the peaks observed at 1117 and 1099 cm^{-1} represented the stretching vibration of the C—O ester groups. Guillen and Cabo (2004) reported that the changes in a ratio of A_{2854}/A_{1237}, A_{2854}/A_{1163}, A_{2854}/A_{1117} or A_{2854}/A_{1099} could be used to indicate the oxidation degree of the samples. After 15 days of storage, seabass sample exhibited the greater changes of these ratios than did red tilapia sample. The lower ratio of A_{2854}/A_{1237} which represented C—O—C in ethers and esters were found in both samples after 15 days of storage. The result suggested that the advanced lipid oxidation and lipid hydrolysis could be occurred in fish muscles with increasing storage time.

A


Seabass, Day 0
Seabass, Day 15
Absorbance
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm-1)

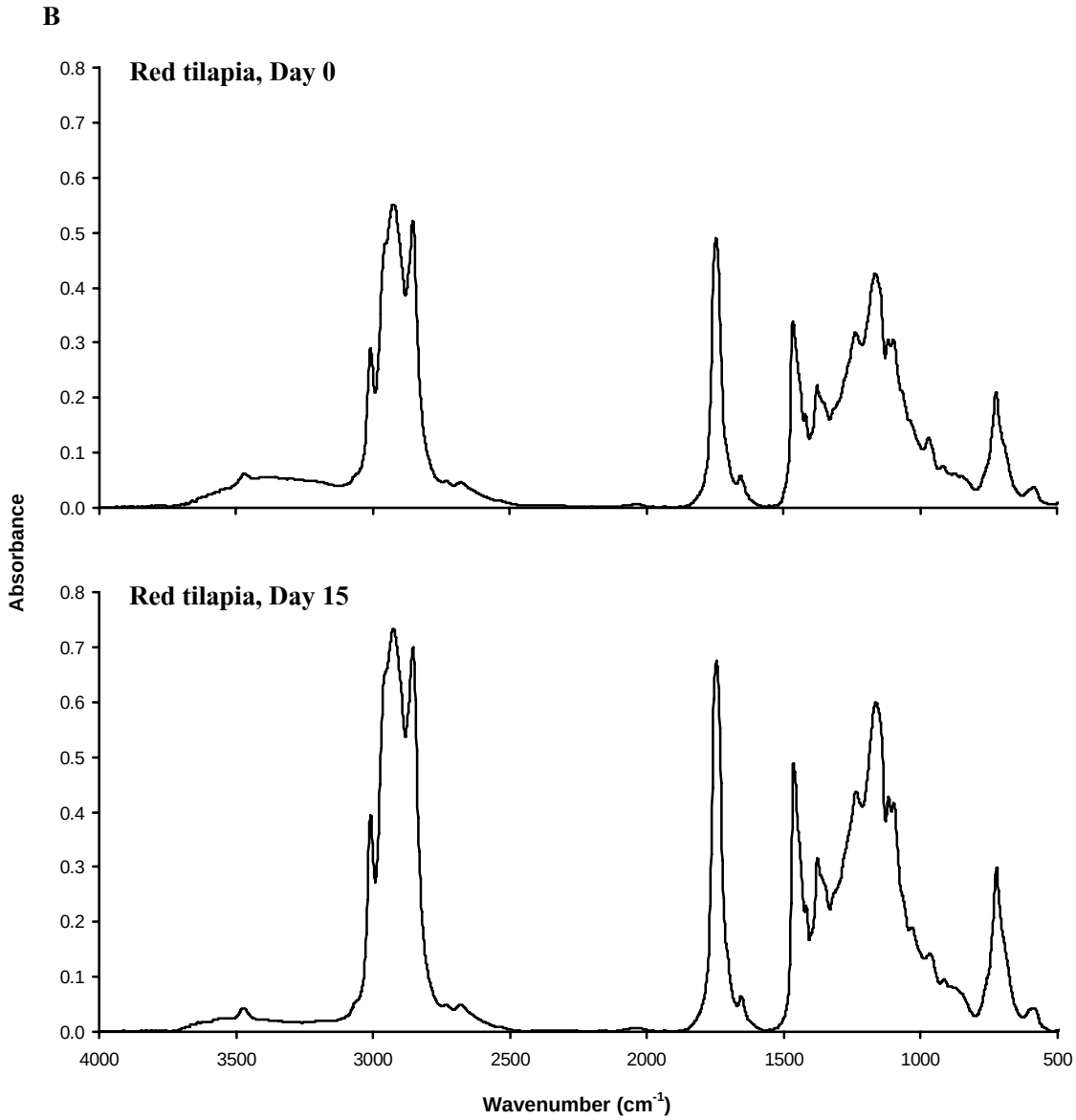


Figure 10. FTIR spectra of crude oil extracted from fresh and 15 days-ice-stored seabass (A) and red tilapia muscles (B)

2.4.7 Sensory property of seabass and red tilapia muscle during iced storage

Fresh seabass and red tilapia were generally considered to have very high acceptability for consumers. However, fresh fish is susceptible to spoilage caused by both microbiological and chemical reactions. Changes in odor intensities of seabass and red tilapia muscles during 15 days of iced storage are shown in Figure 11. Both samples possessed off-odors including fishy, rancid and spoiled odors as storage time increased. Within the first 6 days of storage, no fishy and rancid odors were found in seabass muscle. Spoiled odor was detected in seabass muscle at day 12 of storage ($P < 0.05$). Similar results were observed in red tilapia muscle. In general, seabass muscle exhibited the higher intensity of off-odors, both fishy and rancid, than did red tilapia muscle. This was most likely owing to the higher content of PUFA in seabass in comparison with red tilapia sample (Table 4). Fishy and rancid odors of both samples were more intense after day 6 and dramatically increased up to 15 days of storage ($P < 0.05$). Kyrana and Lougovois (2002) reported that off-flavors of European seabass were evident after 15 days of storage in melting ice.

The formation of TMA depends on the content of TMAO primarily found in marine fish and results in a detectable fishy odor (Gram and Huss, 1996). Since there was very low amount of TMA in seabass (0.33 mg N/100 g sample), a brackish-water fish and undetectable amount of TMA in red tilapia, a fresh-water fish, it could be presumed that the fishy odors developed during storage of both fish species were not mainly the results of TMA. It was most likely that the development of off-odor, rancid and fishy, in seabass and red tilapia muscles during 15 days of iced storage were mainly attributed to lipid oxidation.

The detectable fishy and rancid odors of both muscles were related with the marked increase in TBARS after 6 days of iced storage (Figure 8C). In addition, the increased peak amplitude representing aldehyde was found in both fish muscles after keeping for 15 days (Figure 10). The formation of the secondary lipid oxidation products is one of the main causes of the development of undesirable odors in fish flesh. Human olfactory receptors usually have remarkably low organoleptic thresholds to most of these volatile compounds (McGill *et al.*, 1977). Therefore, the higher the lipid oxidation, the higher off-odor could be obtained. Sohn *et al.* (Sohn *et al.*, 2005) reported the correlations between total lipid hydroperoxide content and the intensity of fishy as well as rancid off-odor in dark muscles of yellowtail during iced storage.

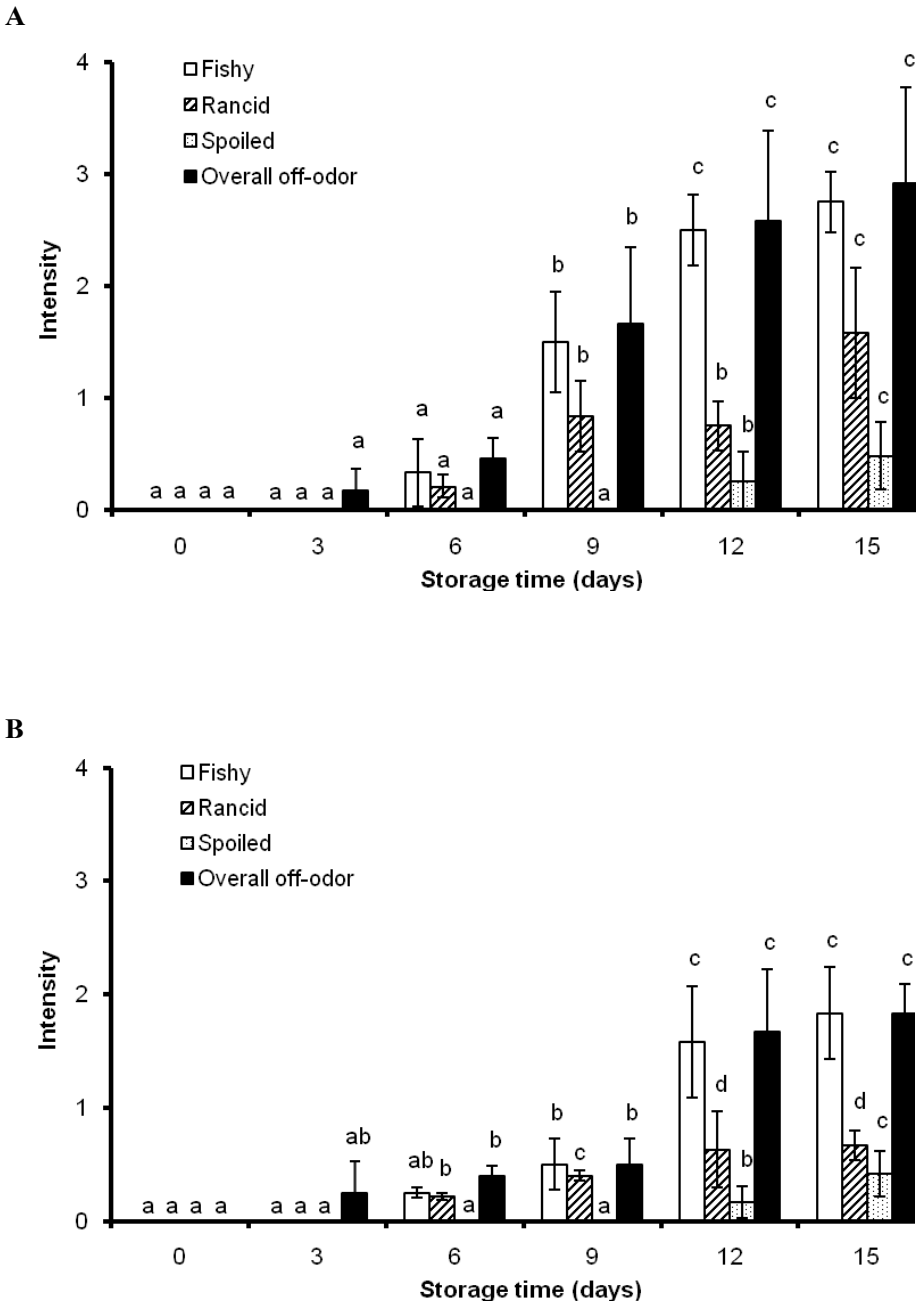


Figure 11. Changes in odor intensity of seabass (A) and red tilapia (B) muscles during iced storage. Score ranges from none (score=0) to strong (score=4). Different letters within the same odor indicate significant differences ($P < 0.05$). Bars represent the standard deviation (n=3)

However, these correlations were not found in ordinary muscles of yellowtail. Furthermore, the oxidation of Mb is possibly important factor associated with accelerated lipid oxidation and the generation of off-odor. Lee *et al.* (2003) reported that surface metMb and TBARS formations of refrigerated tuna steak increased upon 6 days of storage and were associated with a decrease in odor acceptability. The remarkable increases in fishy odor for both fish could be observed after 9 days of iced storage. However, fishy odor was more predominant than rancid odor at all storage times tested. Therefore, the changes of Mb and lipid oxidation were considered to relate with a development of undesirable odor, particularly fishy odor in both fish species during iced storage.

2.5 Conclusion

During iced storage, seabass and red tilapia muscles underwent lipid oxidation and lipid hydrolysis along with the oxidation of Mb and the release of free iron. As storage time increased, the rancid and fishy odors in fish muscles were developed to a higher extent. Fishy odor development found in seabass and red tilapia was not mainly a result of TMA formation, but was associated with lipid oxidation. Therefore, the suppression of oxidation of Mb and lipid could be a means to maintain the quality of both fish during iced storage. A model system to assess the impact of Mb on lipid oxidation should be further conducted.

CHAPTER 3

Isolation, characterization and stability of myoglobin from Eastern little tuna (*Euthynnus affinis*) dark muscle

3.1 Abstract

This study aimed to purify and characterize myoglobin (Mb) from dark muscle of Eastern little tuna (*Euthynnus affinis*). Mb purified by ammonium sulfate precipitation (65-100% saturation), followed by Sephadex G-75 chromatography had a molecular weight of 15,680 Da. pI of both oxyMb and metMb was estimated to be 5.25 as determined by zeta potential analysis. Transition temperatures were 61 and 60ºC for oxyMb and metMb, respectively. The color values (L^*, a^* and b^*) and absorption spectra of Mb solutions differed significantly, depending upon the form of Mb. OxyMb and metMb had the Soret bands at 413 and 407 nm, respectively. pH and thermal stability of Mb was tested under the pH range of 3-11 and temperature range of 4-70ºC, respectively. Loss of heme-globin complex and autoxidation were dominant at pH 3 as evidenced by the disappearance of Soret band and formation of metMb. Heating at temperature above 60ºC had a great impact on Mb denaturation. With increasing temperature and incubation time, oxyMb was susceptible to oxidation and conformational change, while metMb tended to be more stable. Thus, the form of Mb governed its properties and stability.

3.2 Introduction

Myoglobin (Mb) has been known to be a major contributor to the color of muscle, depending on its redox states (deoxyMb, oxyMb and metMb) and concentration (Faustman and Cassens, 1990). Furthermore, Mb has a close relationship with lipid oxidation which could influence the deterioration in food. The lipid oxidation in fish muscle was promoted

by the oxidation of Mb, which played a considerable role in the generation of hydroperoxides (Sohn *et al.*, 2005).

Mb is made up of a single polypeptide chain, globin, and a prosthetic heme group, an iron (II) protoporphyrin-IX complex (Pegg and Shahidi, 1997). The molecular weight of Mb is determined to be 14-18 kDa and fish Mb is generally smaller than mammalian counterpart (Chaijan *et al.*, 2007; Fosmire and Brown, 1976; Satterlee and Zachariah, 1972; Ueki and Ochiai, 2004; Yamaguchi *et al.*, 1979). The stability of Mb varies with species due to different amino acid sequences and secondary structure of globin. Chaijan *et al.* (2007) demonstrated that sardine Mb was prone to oxidation and denaturation at temperature above 40°C and at very acidic or alkaline pHs. Moreover, the rate of Mb autoxidation was related to oxygen concentration (Faustman and Cassens, 1990). The stability of Mb which affects color as well as lipid oxidation of meat is associated with its autoxidation rate. Tuna has been known to provide one of the most remarkable red muscle, in which Mb is found at high concentrations (Chow *et al.*, 2004; Ueki and Ochiai, 2004). To obtain the fish with high quality during the storage, the suppression of Mb oxidation is required.

Tuna and tuna products are widely consumed in many parts of the world because they are rich in proteins. Tuna viscera were used as the raw materials for fish sauce production (Dissaraphong *et al.*, 2006). Additionally, tuna head has been used as the important source of fish oil containing a high amount of ω-3 fatty acid (Chantachum *et al.*, 2000). Eastern little tuna (*Euthynnus affinis*) is a species available in the Gulf of Thailand and the Indian Ocean and provides the high global economic value as a low-mercury tuna for canning and sashimi (Fisheries Foreign Affairs Division, 2007; Hajeb *et al.*, 2009). However, no information regarding the Mb from Eastern little tuna has been reported. The objective of this investigation was to purify and characterize the Mb from Eastern little tuna dark muscle and to study the properties and stability of Mb over a wide range of pHs and temperatures.

3.3 Materials and Methods

3.3.1 Chemicals

Sodium dodecyl sulfate (SDS), β-mercaptoethanol (βME), Triton X-100, pyridine, bathophenanthroline disulfonic acid and wide range protein markers were purchased from Sigma (St. Louis, MO, USA). Acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED) and bis-acrylamide were purchased from Fluka (Buchs, Switzerland). Sodium dithionite was obtained from Riedel (Seeize, Germany). Sodium chloride, trichloroacetic acid, sodium nitrite and iron standard solution were procured from Merck (Damstadt, Germany).

3.3.2 Fish samples

Five Eastern little tuna (*E. affinis*) with the average weight of 0.5-0.55 kg were obtained from the dock in Songkhla province, Thailand. The fish were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla. The fish were immediately washed, filleted and manually excised. The dark muscles were collected, pooled as the composite sample and minced until the uniformity was obtained. The mince was kept in ice within 1 h prior to Mb extraction.

3.3.3 Purification of Mb from Eastern little tuna

Extraction and purification of Mb was performed according to the method of Trout and Gutzke (1996) with a slight modification. Eastern little tuna mince (100 g) was mixed with 300 ml of cold extracting medium (10 mM Tris-HCl, pH 8.0 containing 1 mM EDTA and 25 g/l Triton X-100). The mixture was homogenized for 1 min at a speed of 13,000 rpm using an IKA Labortechnik homogenizer (Selangor, Malaysia). After centrifugation at 9,600*g* for 10 min at 4ºC using an Avanti J-E centrifuge (Beckman Coulter, Palo Alto, CA, USA), the supernatant was filtered through a Whatman No. 4 filter paper. The filtrate referred to as "crude Mb extract" was subjected to ammonium sulfate fractionation (65-100% saturation). The precipitate obtained was dissolved in a minimal volume of cold 5 mM Tris-HCl buffer, pH 8.5, which was referred to as "starting buffer". The mixture was then dialyzed against 10 volumes of the same buffer with 20 changes at 4ºC. The dialysate was immediately applied onto a Sephadex G-75 column (2.6 × 70

cm; Amersham Bioscience, Uppsala, Sweden) which was equilibrated with the starting buffer. The separation was conducted using the starting buffer at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and measured at 280 and 540 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The fractions with the high absorbance at 540 nm were pooled and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

3.3.3.1 SDS-PAGE

The purity and molecular weight (MW) of Mb were determined by SDS-PAGE according to the method of Laemmli (1970). Protein samples were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% SDS, 20% glycerol and 10% βME). Samples were loaded onto polyacrylamide gels comprising 4% stacking gel and 17.5% running gel and subjected to electrophoresis at a constant current of 15 mA/gel using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, the gel was stained with 0.02% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 25% (v/v) methanol and 10% (v/v) acetic acid. The wide range markers [myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase (97 kDa), fructose-6-phosphate kinase (84 kDa), albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa) and aprotinin (6.5 kDa)] were used for MW estimation.

3.3.3.2 Preparation of oxyMb and metMb

OxyMb and metMb were prepared according to the method of Tang *et al.* (2004) with some modifications. An aliquot (5 ml; 2 mg protein/ml) of purified Mb was converted to oxyMb by the addition of 50 mg of sodium dithionite. MetMb was prepared by adding 50 mg of potassium ferricyanide to 5 ml of the purified Mb solution. The sodium dithionite and potassium ferricyanide were removed by dialysis of the sample against 10 volumes of cold 50 mM phosphate buffer, pH 7.0 with 20 changes of dialysis buffer. Protein concentrations of dialysates, representing oxyMb and metMb, were adjusted to 0.2 mg protein/ml.

3.3.3.3 Determination of protein concentration

Protein concentrations of Mb solutions were determined by the Lowry method (Lowry and Tinsley, 1976). Bovine serum albumin was used as the protein standard.

3.3.4 Characterization of Eastern little tuna Mb

3.3.4.1 Color

The color of oxyMb and metMb solutions (0.2 mg protein/ml) were measured and expressed as L^*, a^* and b^* values using a colorimeter (Juki Corp, Tokyo, Japan). L^*, a^* and b^* values represented lightness, redness/greenness and yellowness/blueness, respectively.

3.3.4.2 Absorption spectra

The absorption spectra of oxyMb and metMb solutions were taken using a spectrophotometer. The spectra were recorded from 350 to 750 nm at the scanning rate of 1000 nm/min using 50 mM phosphate buffer, pH 7.0 as a blank.

3.3.4.3 Proportion of Mb forms

The proportions of three Mb forms; deoxyMb, oxyMb and metMb were calculated by a modified Krzywicki's equations (Tang *et al.*, 2004) as follows:

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

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

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

where $R_1 = A_{582}/A_{525}$, $R_2 = A_{557}/A_{525}$ and $R_3 = A_{503}/A_{525}$.

3.3.4.4 Differential scanning calorimetry (DSC)

DSC thermograms of oxyMb and metMb solutions were determined according to the method of Chaijan *et al.* (2007) using Perkin Elmer Differential Scanning Calorimeter (DSC) (Model DSC-7, Norwalk, CT, USA). Temperature calibration was performed using the Indium thermogram. The specimen (about 12 mg) was accurately weighed, placed into an aluminium pan and sealed. The scanning was performed at a heating rate of 2ºC/min over the temperature range of 20-100ºC. The iced water was used as a cooling medium and the system was equilibrated at 20ºC for 5 min prior to the scan. The empty aluminium pan was used as a

reference. The temperature at each peak maximum was recorded as the maximum transition temperature (T_m) and total denaturation enthalpy (ΔH).

3.3.4.5 Zeta potential analysis

Zeta (ζ) potential of oxyMb and metMb solutions were measured by Zeta potential analyzer model ZetaPALs (Brookhaven Instruments Co., Holtsville, NY, USA). Solutions (20 ml) were transferred to autotitrator model BI-ZTU (Brookhaven Instruments Co., Holtsville, NY, USA), in which pH of solutions were adjusted to 2-12 using either 1.0 M nitric acid or 1.0 M KOH. The obtained zeta potential of solution at all pHs was recorded.

3.3.5 Effect of pH and temperature on the properties and stability of Eastern little tuna Mb

The solutions of oxyMb and metMb were dialyzed against 10 volumes of cold 50 mM phosphate buffers with various pH values (pHs 3, 5, 7, 9 and 11) for 15 h according to the method of Chaijan *et al.* (2007). The protein concentration of Mb solutions was adjusted to 0.2 mg/ml with the corresponding buffer. All the procedures were carried out at 4ºC.

Thermal stability of oxyMb and metMb was studied. Three ml of oxyMb and metMb solution was pipetted into a test tube and subjected to the incubation at 4, 20, 30, 40, 50, 60 and 70ºC in an ice bath or a temperature controlled water bath (Memmert, Schwabach, Germany) for 10, 30 and 60 min according to the method of Chaijan *et al.* (2007). At the designated time, the samples were taken for analyzes.

3.3.5.1 Heme protein degradation and Mb oxidation

Heme protein degradation of the treated solutions was determined by the changes in absorption spectra and Soret band (Swatland, 1989). To determine the oxidation of Mb, the proportions of three Mb forms; deoxyMb, oxyMb and metMb were calculated following a modified Krzywicki's equations (Tang *et al.*, 2004).

3.3.5.2 Mb aggregation ratio

The treated Mb solutions were filtered through a Millipore filter (pore size, 0.45 μm) (Millipore, Bedford, MA, USA). The filtrate was determined for the absorbance at 280 nm. The percentage of the absorbance decrement to the untreated original absorbance was calculated as Mb aggregation ratio (Chow *et al.*, 2004).

3.3.5.3 Tryptophan fluorescence

To monitor the changes in protein structure, tryptophan fluorescence of treated Mb solutions was measured with a RF-1501 fluorometer (Shimadzu, Kyoto, Japan) at an excitation wavelength of 280 nm and an emission wavelength of 325 nm according to the method of Chanthai *et al.* (1996).

3.3.5.4 Non-heme iron content

Non-heme iron content of treated Mb solutions was determined according to the method of Schricker *et al.* (1982) with a slight modification. One ml of oxyMb and metMb were pipetted into a screw cap test tube and 50 µl of 0.39% (w/v) sodium nitrite were added. Four ml of a mixture of 40% trichloroacetic acid and 6 M HCl (ratio of 1: 1 (v/v), prepared freshly) were added. The tightly capped tubes were incubated at 65ºC for 22 h and then cooled at room temperature for 2 h. The supernatant (400 µl) was mixed with 2 ml of the non-heme iron color reagent, a mixture of bathophenanthroline disulfonate reagent (0.162 g of bathophenanthroline disulfonic acid + 100 ml of double-deionized water + 2 ml of thioglycolic acid), double-deionized water and saturated sodium acetate solution at a ratio of 1:20:20 (v/v/v), prepared freshly. After vortexing and standing for 10 min, the absorbance was measured at 540 nm. The non-heme iron content was calculated from iron standard curve. The iron standard solutions ($Fe(NO_3)$ in HNO_3), with the concentrations ranging from 0 to 2 ppm, were used.

3.3.6 Statistical analysis

Experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

3.4 Results and Discussion

3.4.1 Purification of Mb from Eastern little tuna dark muscle

Crude Mb extract from Eastern little tuna dark muscle was subjected to ammonium sulfate precipitation, followed by gel filtration using the Sephadex G-75 column. After being chromatographied using Sephadex G-75 column, two major peaks exhibiting A_{280} were obtained (Figure 12A). The second peak had the brown color, most likely containing Mb as the major component.

During purification, crude Mb extract and its fractions, 65-100% ammonium sulfate and Sephadex G-75 fractions, were determined for protein patterns using SDS-PAGE as shown in Figure 12B. Crude Mb extract contained a large number of proteins with the MW greater than 36 kDa (Figure 12B, lane 2). After ammonium sulfate precipitation (65-100% saturation), proteins with MW higher than 84 kDa were discarded. Coincidentally, the band intensity of proteins with MW lower than 36 kDa, especially with MW of 15 kDa, became more intense (Figure 12B, lane 3). The contaminating proteins with MW higher than 15 kDa could be further removed by sephadex G-75 gel filtration chromatography. Pooled fractions (fraction No. 72 to 82) showed the single band on the SDS-PAGE, suggesting the homogeneity of purified protein. MW of Eastern little tuna Mb was estimated to be 15,680 Da. The obtained MW was similar to that of another tuna species including bigeye tuna (15,540 Da) and yellowfin tuna (16,000 Da), but was larger than that of mackerel (14,900 Da) and sardine (14,600 Da) (Fosmire and Brown, 1976; Ueki and Ochiai, 2004; Yamaguchi *et al.*, 1979). MW of Mb from porcine, ovine and sperm whale were 17,700, 17,100 and 17,000 Da, respectively. MW of fish Mb is generally smaller than that of mammalian counterpart (Satterlee and Zachariah, 1972).

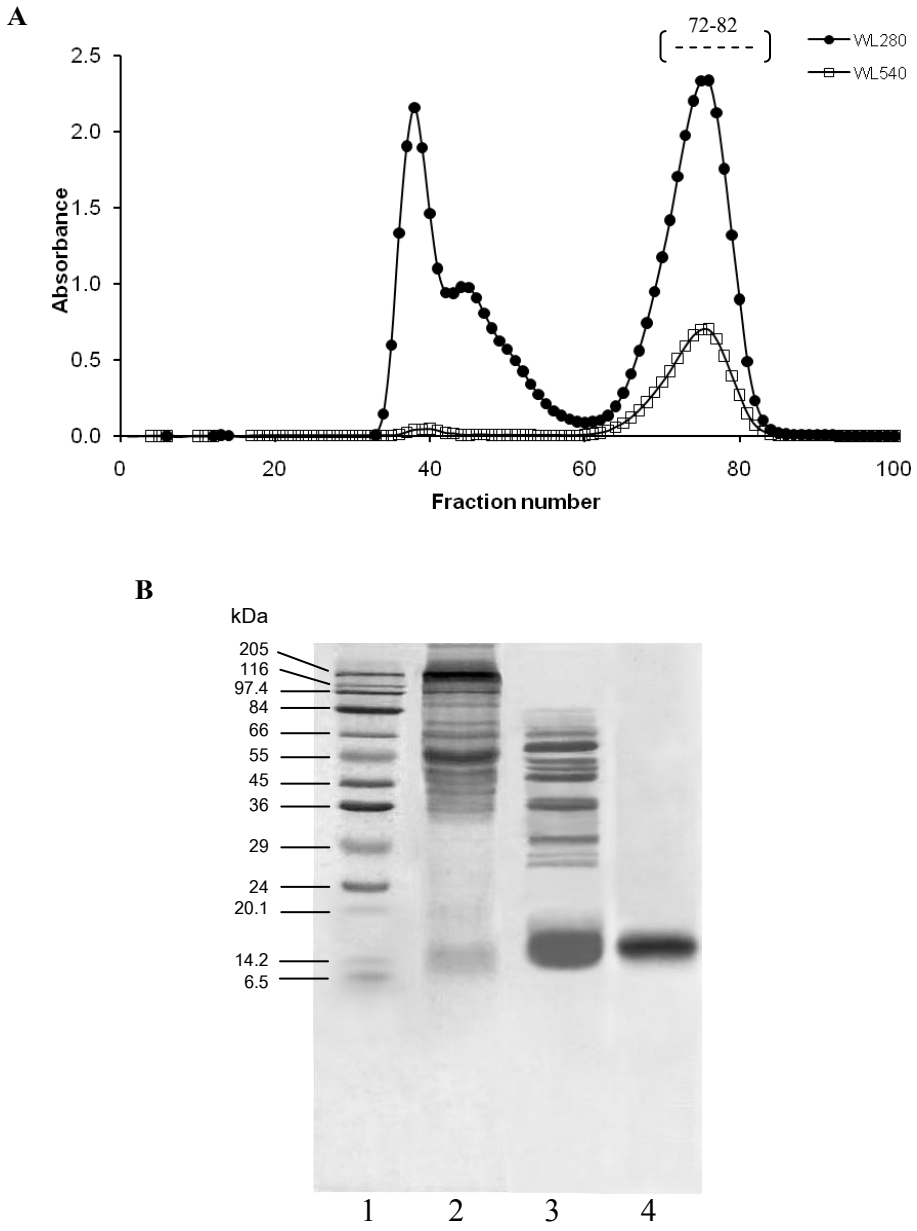


Figure 12. Elution profile of Mb from Eastern little tuna dark muscle on the Sephadex G-75 (A) and SDS-PAGE pattern of crude Mb extract and its fractions (B). Lane 1, wide range markers; lane 2, crude Mb extract; lane 3, ammonium sulfate precipitated fraction; lane 4, Sephadex G-75 fraction (fraction No. 72 to 82)

3.4.2 Color and absorption spectra of oxyMb and metMb

The colors of Mb from Eastern little tuna with a concentration of 0.2 mg protein/ml were expressed as L^* (lightness), a^* (redness) and b^* (yellowness) values which were 79.12, 11.11 and 33.30 for oxyMb solution and 82.72, 4.22 and 38.85 for metMb solution, respectively. Higher a^* value and lower b^* value of oxyMb solution in comparison with metMb solution ($P < 0.05$) indicated that oxyMb was more reddish than metMb. Mb autoxidation was found to cause an increase in L value and a decrease in a value (Chen and Chow, 2001). Therefore, a higher L^* value with the coincidentally lower a^* value in metMb solution suggested the autoxidation of Mb. The color of fresh meat is governed by the relative amounts of three forms of Mb; deoxyMb, oxyMb and metMb (Lindahl *et al.*, 2001). DeoxyMb was the purple pigment of deep muscles. Upon the exposure to air, Mb combines with oxygen to form the bright red oxyMb, which is considered attractive by the consumer. However, the contact of Mb with oxygen also leads to the formation of the oxidized form, metMb, which is brown or gray and rather unattractive (Faustman and Cassens, 1990).

The absorption spectra of oxyMb and metMb solutions from dark muscle of Eastern little tuna are depicted in Figure 13A and 13B. The intense peaks in a blue region (350-450 nm) corresponding to the Soret bands were found at 413 and 407 nm for oxyMb and metMb solutions, respectively. Soret bands of oxyMb and metMb purified from tuna (*Thunnus albacares*) were found at 414 and 406 nm (Smulevich *et al.*, 2007); and the bands of oxyMb and metMb from meat were noticeable at 416 and 410 nm, respectively (Swatland, 1989). Soret band results mainly from the interaction of the heme moiety with apoMb, hence it can be used to monitor the unfolding of hemoproteins (Chen and Chow, 2001). In a region of 450-750 nm, the peaks were found at wavelengths of 542 and 577 nm for oxyMb solution and 502 and 630 nm for metMb solution. The similar spectra patterns were reported for equine heart Mb which had the peak wavelengths at 544 and 582 nm for oxyMb; and 503 and 632 nm for metMb (Tang *et al.*, 2004). Tang *et al.* (2004) demonstrated that 3 redox forms of Mb resulted in individually different absorption spectra which had the isobestic point at 525 nm. The maximum wavelengths at 503, 557 and 582 nm representing metMb, deoxyMb and oxyMb, respectively, have been used for determining the relative proportions of Mb redox forms in aqueous solution (Tang *et al.*, 2004). OxyMb solution had the highest proportions of oxyMb (61.88%), followed by metMb (30.45%)

and deoxyMb (7.67%), respectively. This indicated that the oxidation of oxyMb to metMb more likely occurred during preparation of oxyMb solution. For metMb solution, metMb (91.47%) was dominant and deoxyMb (4.53%) and oxyMb (4.00%) were found at the lower levels.

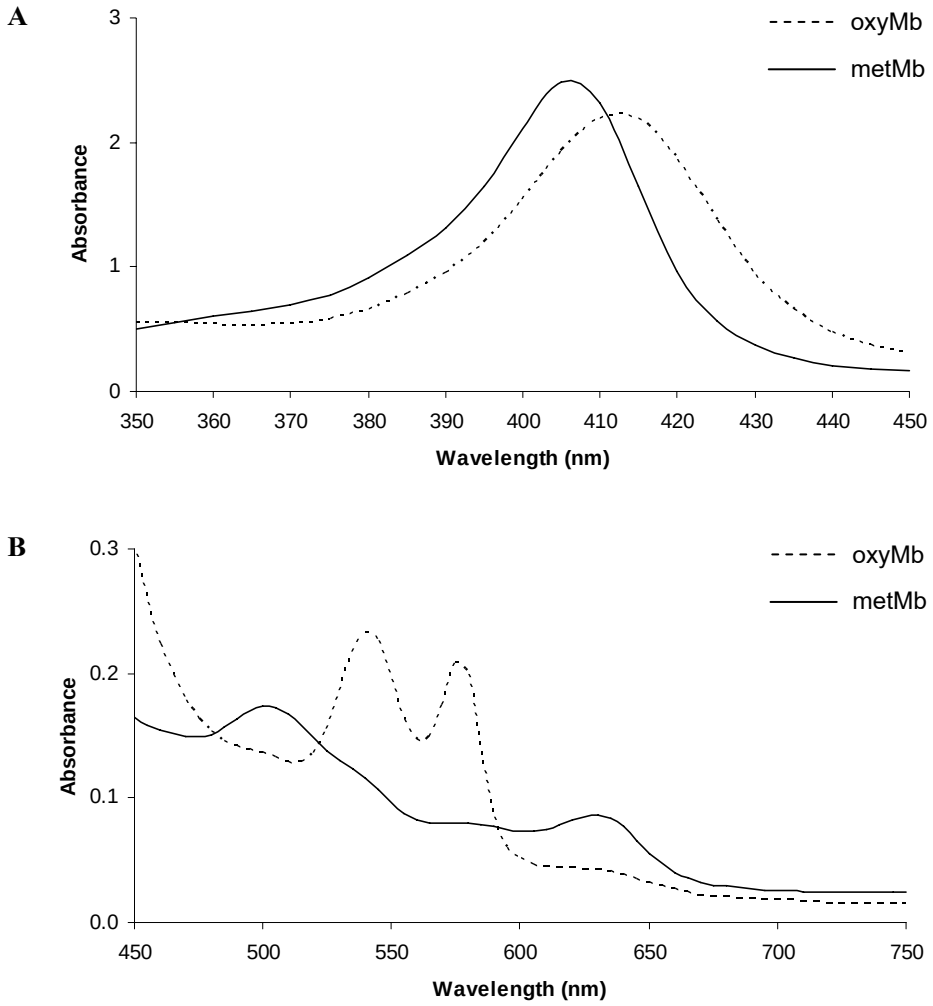


Figure 13. The absorption spectra in a region of 350-450 nm (A) and a region of 450-750 nm (B) of oxyMb and metMb from Eastern little tuna dark muscle. Bars represent the standard deviation (n=3)

3.4.3 Differential scanning calorimetry (DSC) of oxyMb and metMb

Transition temperatures (T_m) and total denaturation enthalpy (ΔH) of oxyMb and metMb were analyzed by DSC. T_m of 61 and 60ºC and ΔH of 0.105 and 0.519 J/g were found for oxyMb and metMb, respectively. Chen *et al.* (2004) reported that T_m of Mb from migratory, demersal, aquaculture and elasmobranch fish species were in a range of 46.4-71.8ºC. Thermal stability of Mb varies with fish species due to different amino acid sequence and secondary structure of globin (Chen *et al.*, 2004; Colonna *et al.*, 1983). The low conformational stability of fish Mb in comparison with mammal Mb has been reported (Chanthai *et al.*, 1996; Colonna *et al.*, 1983). A slight difference in T_m was observed between oxyMb, the ferrous form, and metMb, the ferric form. For bovine Mb, T_m were 74.0ºC for oxyMb, 73.7ºC for deoxyMb and 73.3ºC for metMb (Sepe *et al.*, 2005). For sardine Mb, metMb was most easily denatured by heat when compared with deoxyMb and oxyMb (Chaijan *et al.*, 2007). Thus, oxygen or water molecule which could bind with iron within porphyrin ring might contribute to the conformational stability for each form of Mb.

3.4.4 Zeta potential of oxyMb and metMb

Zeta potential representing the surface charge of oxyMb and metMb from Eastern little tuna is shown in Figure 14. A similar surface charge was observed for both oxyMb and metMb over a whole pH range (pH 2-12) ($P > 0.05$), most likely due to the same amino acid composition of globin. Both forms of Mb exhibited the positive charges at acidic pH, while the negative charges were obtained at pH greater than 5.25. The differences in surface net charge at different pH were governed by the protonation or deprotonation of amino acids, particularly localized on the surface of molecules. When pH is close to isoelectric point (pI), net charge of protein is zero (Copeland, 1994; Pace *et al.*, 2004). Zero net charge of both forms of Mb was observed at pH 5.25, presumably being the pI of Mb from this species. Mackerel and sardine Mbs had pI of 5.8-5.9, while horse Mb had pI of 8.1 (Shiraki *et al.*, 2002; Yamaguchi *et al.*, 1979).

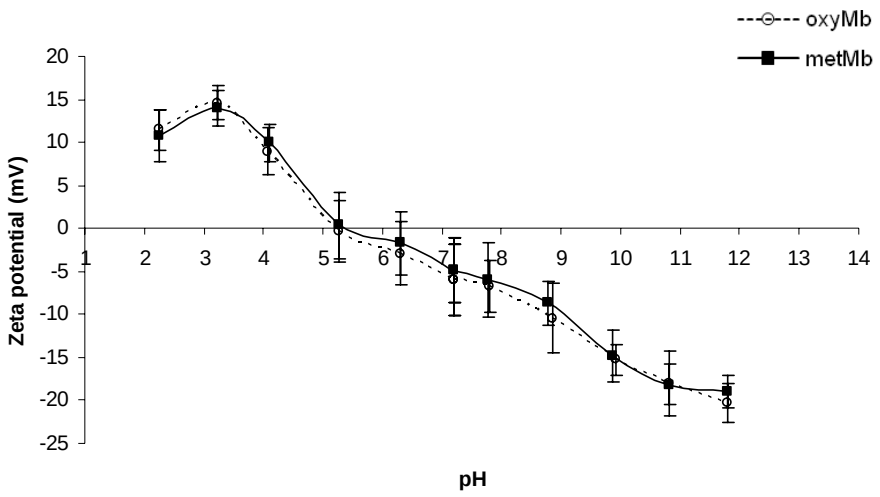


Figure 14. Zeta potential of oxyMb and metMb from Eastern little tuna dark muscle. Bars represent the standard deviation (n=3)

3.4.5 Effect of pH on properties and stability of oxyMb and metMb

The absorption spectra of oxyMb and metMb from Eastern little tuna at varying pH are displayed in Figure 15. In a region of 350-450 nm, oxyMb and metMb had the disappeared Soret bands at pH 3, indicating a weak heme-globin complex at extremely acidic pH. Low pH could reduce the stability constant for heme-globin linkage and increase the autoxidation rate (Chaijan *et al.*, 2007; Renerre *et al.*, 1992). At pH 3, the proportions of metMb form were highest for both oxyMb solution (67.87%) and metMb solution (96.14%). This result confirmed the autoxidation as indicated by metMb formation at acidic pH. The Soret bands were noticed over a pH range of 5-11 for both forms of Mb. However, the peaks of both forms had the decrease in area and shifted to the higher wavelength as pH increased. These pointed out a degradation of heme structure and a change of Mb into a reduced form under alkaline condition. On the other hand, the neutral pH yielded a compact and unique spatial structure of Mb (Chen *et al.*, 2004). When pH increased from 5 to 11, the proportions of oxyMb were increased from 25.31 to 57.69% for oxyMb and from 4.32 to 25.86% for metMb. Phillips Jr *et al.* (1999) reported the occurrence of hydroxyl-metMb at high pH which appears to be similar to oxyMb. In a pH range of 5-9, oxyMb exhibited a greater change in absorption spectra than metMb. This indicated a less stability of oxyMb, in comparison with metMb in those pH ranges.

For absorption spectra of both Mb forms in a region of 450-750 nm, oxyMb peak was dominant at pH ≥7, indicating the stability of oxyMb at neutral to alkaline pHs (Figure 15B). For metMb solution, the typical absorption spectra of metMb were found at pH 5 and 7, suggesting the stability of metMb at neutral to slightly acidic pH (Figure 15D). According to the Bohr's effect, the proton-catalyzed displacement process was mainly responsible for promoting the autoxidation of Mb at acidic pHs, while the oxygenation of Mb might occur at alkaline pHs (Renerre *et al.*, 1992).

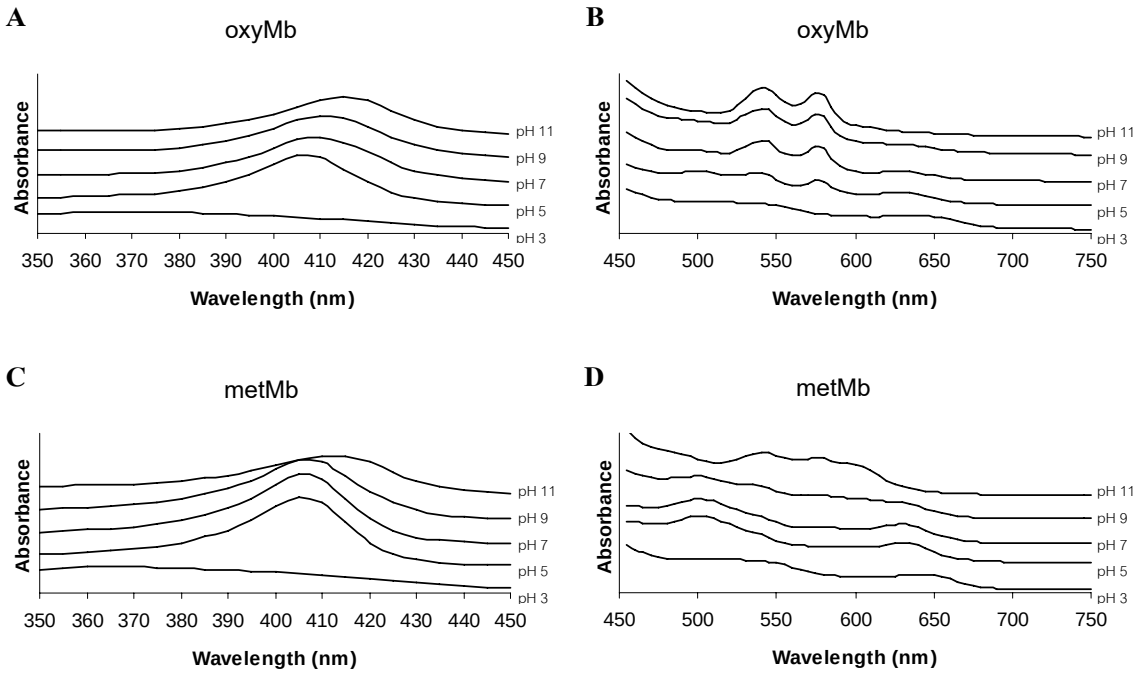


Figure 15. Effect of pH on the absorption spectra in a region of 350-450 and 450-750 nm of oxyMb (A and B) and metMb (C and D) (4°C) from Eastern little tuna dark muscle

Tryptophan fluorescent intensity for oxyMb and metMb from Eastern little tuna in a pH range of 3-11 is shown in Figure 16A. Tryptophan is involved in forming favorable and specific tertiary interactions in the native apoMb structure (Ivana *et al.*, 2000). The fluorescence properties of the tryptophan residues can be used to determine the polarity of its local environment and the unfolding of proteins (Copeland, 1994). For both oxyMb and metMb, the highest intensity in tryptophan fluorescence was noticeable at pH 3 ($P < 0.05$). At acidic pH, an enhanced protonation of Mb might induce the conformational changes as well as unfolding of apoMb as evidenced by an increase in fluorescent intensity. Acid-denatured Mb is associated with protein unfolding and the loss in heme group (Chaijan *et al.*, 2007). At pH 5, which was close to pI of Mb from Eastern little tuna, the heme-globin complex was compacted and stabilized. However, decreases in tryptophan fluorescent intensity were observed as pH increased up to pH 11 ($P < 0.05$), suggesting the stability of both Mb forms at very alkaline pH. This result was in agreement with the highest peak amplitude in absorption spectra (Figure 15). Pace *et al.* (2004) reported that hydrophobic clusters and other native-like structures of protein could exist even under strongly denaturing conditions. The similar behavior was previously reported in sardine Mb which exhibited the highest fluorescent intensity at very acidic pH and the decreased intensity was found at neutral to alkaline pH (Chaijan *et al.*, 2007). Tryptophan fluorescent intensity of oxyMb was higher than that of metMb when tested at pH range of 5-11 ($P < 0.05$). The result suggested that oxyMb, ferrous form, was more susceptible to unfolding or conformational changes than metMb, ferric form.

Aggregation ratio of oxyMb and metMb at varying pH is depicted in Figure 16B. The highest aggregation ratio for both Mb solutions was observed at pH 5, which was 24.60 and 26.14% for oxyMb and metMb, respectively ($P < 0.05$). This indicated the most compactness of heme-globin complex at pH close to pI (5.25) of purified Mb from Eastern little tuna. At pI of protein, the charges on protein surface are generally arranged so that there are more attractive than repulsive interactions (Pace *et al.*, 2004). Aggregation ratio for both Mb solutions was decreased when pH was far from pH 5 and showed the lowest values at pH 11 (5.20% for oxyMb and 9.19% for metMb) ($P < 0.05$). The improved charges of proteins under the pH far from pI contribute favourably to the repulsive interactions, which destabilize the native states of proteins (Pace *et al.*, 2004). At pH above 5, a higher aggregation ratio was found in metMb solution ($P <$

0.05). The native state of Mb is characterized by a tightly folded conformation with a heme group bound to the protein in a hydrophobic pocket (Pegg and Shahidi, 1997). The conformational changes of apoMb induced by hydrogen ions or hydroxyl ions could lead a susceptible heme group to destruction or autoxidation (Chaijan *et al.*, 2007; Renerre *et al.*, 1992).

Non-heme iron content for oxyMb and metMb in a pH range of 3-11 is depicted in Figure 16C. No differences in non-heme iron content were found between oxyMb and metMb solutions ($P > 0.05$), suggesting that Mb forms had no effect on iron releasing from heme structure at all pHs tested. For both forms of Mb, no differences in non-heme iron content were observed in a pH range of 5-11 ($P > 0.05$), while the highest non-heme iron content was observed at pH 3 (0.56 ppm for oxyMb and 0.58 ppm for metMb) ($P < 0.05$). These results suggested that the disruption of porphyrin ring probably occurred at very acidic pH, leading to the release of free iron named “non-heme iron”. Normally, three edges of the porphyrin are buried in the protein interior and stabilized by hydrophobic interactions with apolar side chains that line the heme pocket. The fourth edge contains the solvent-exposed heme propionates, which interact electrostatically with polar amino acid side chains located on the surface of the protein (Liong *et al.*, 2001). In accordance with the result of Soret band, the destabilized heme-globin linkage and heme destruction might promote the release of iron from heme for both forms of Mb at pH 3. It was most likely that porphyrin ring in hydrophobic pocket of Mb was stable under neutral and alkaline condition. Tang *et al.* (1998) reported that unfolding of apoprotein and hydration of the heme pocket were the key factors in the cleavage of the iron-histidine bond in Mb.

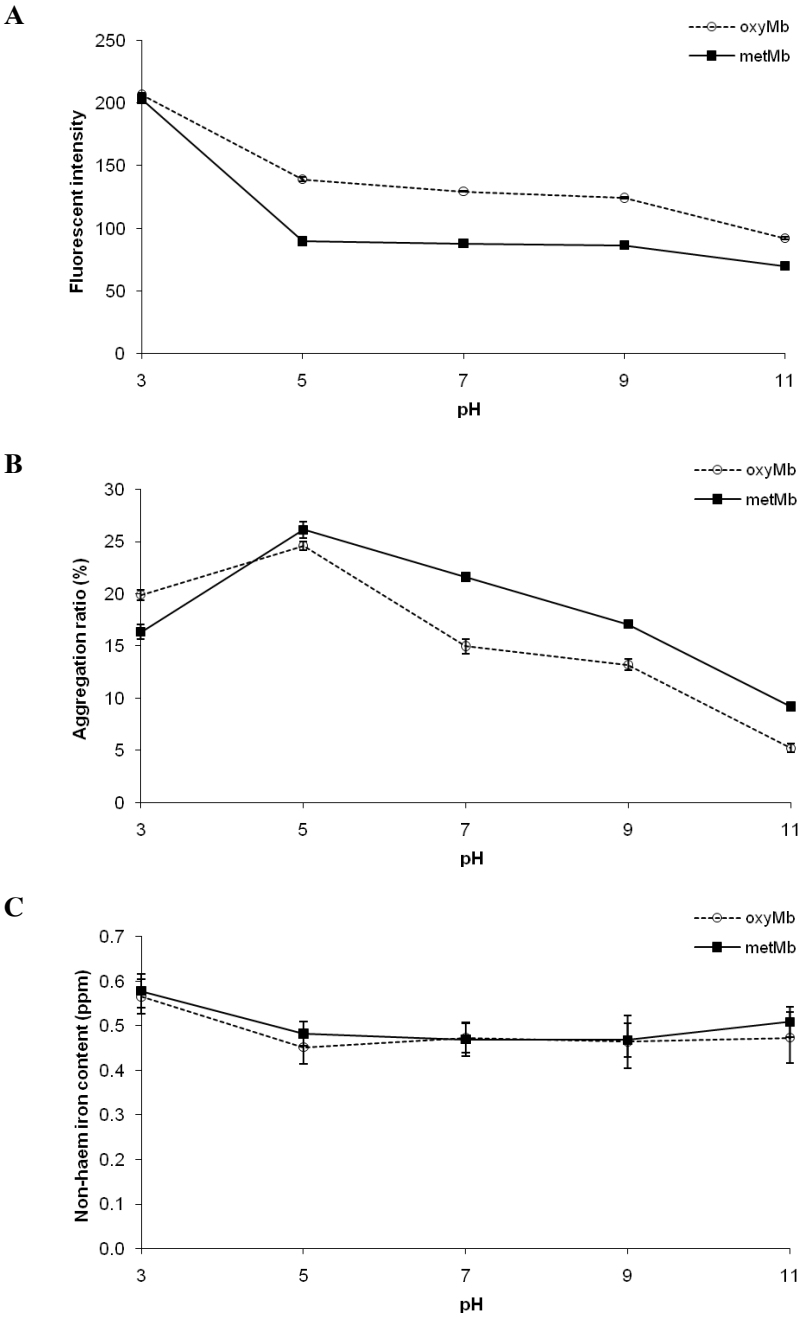


Figure 16. Effect of pH on tryptophan fluorescent intensity (A), aggregation ratio (B) and non-heme iron content (C) at 4ºC of oxyMb and metMb from Eastern little tuna dark muscle. Bars represent the standard deviation (n=3)

3.4.6 Effect of temperature on properties and stability of oxyMb and metMb

OxyMb and metMb from Eastern little tuna were precipitated when the temperature reached 60ºC. This coincided with the result of DSC, in which the transition temperature of Mb from Eastern little tuna was 60-61ºC. Below the denaturation temperature, the denaturation proceeded slowly but it was very fast at temperature above denaturation one (Chen *et al.*, 2004). Absorption spectra of oxyMb and metMb from Eastern little tuna incubated at 4 to 50ºC are shown in Figure 17. A negligible change in the absorption spectra was observed for metMb solution when heated up to 50ºC, for both 350-450 nm and 450-750 nm regions, regardless of heating time, indicating the high stability of metMb. For oxyMb solution, the alteration in absorption spectra was noticeable. With elevated temperature, Soret bands of oxyMb were slightly decreased in intensity and shifted to a lower wavelength, suggesting the changes of oxyMb into an oxidized form. Moreover, the disappearance of oxyMb spectra in a region of 450-750 nm was found when oxyMb was heated at 50ºC for 60 min. As temperature and incubation time increased, the proportions of metMb form in oxyMb solutions increased by 34.64% along with the decrease in oxyMb form by 39.64% ($P < 0.05$). The higher the temperature and time, the higher the autoxidation rate of Mb was obtained (Chaijan *et al.*, 2007).

Tryptophan fluorescent intensity of oxyMb and metMb solutions heated at varying temperatures and times is depicted in Figure 18A. For oxyMb solution, the fluorescent intensities were increased with increasing temperature up to 60ºC ($P < 0.05$), while no changes were noticeable in metMb solution ($P > 0.05$). Both Mb solutions showed a sharp increase in fluorescent intensities when heating temperature was 70ºC ($P < 0.05$). Additionally, the longer the incubation time, the greater the increase in fluorescent intensity was observed ($P < 0.05$). At higher temperature and longer time, unfolding of globin might occur, causing the exposure of tryptophan and tyrosine residues. From the result, oxyMb solution had a greater intensity of tryptophan fluorescence than did metMb solution over the heating temperatures used ($P < 0.05$). Heating brings about a modification of the tryptophan environment in the molecule. The change in the protein structure exposes the heme to the surroundings, thereby increasing pro-oxidative activity of the heated Mb (Kristensen and Andersen, 1997).

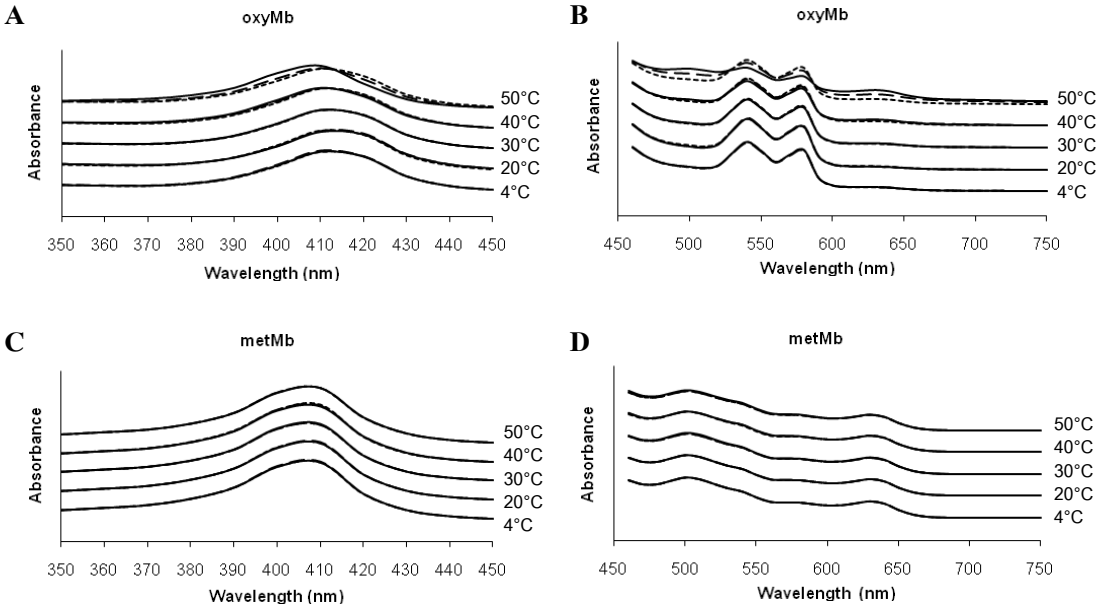


Figure 17. Effect of heating temperatures and time on the absorption spectra in a region of 350-450 and 450-750 nm of oxyMb (A and B) and metMb (C and D) (pH 7.0) from Eastern little tuna dark muscle

Aggregation ratio of oxyMb and metMb solutions subjected to heating at varying temperatures is shown in Figure 18B. For oxyMb solution, a negligible change in aggregation ratio was observed at 4 and 20ºC ($P > 0.05$). At temperature above 20ºC, aggregation ratios increased with increasing temperature and incubation time (4.34 to 82.53%) ($P < 0.05$). For metMb solution, no marked changes in aggregation ratios were noticeable at temperature range of 4-60ºC. The sharp increase in aggregation ratios was found at 70ºC, especially with increasing incubation time (1.97 to 55.75%) ($P < 0.05$). The native state of a protein in solution is characterized by a highly specific and tightly folded conformation. With increasing temperature and longer time, the subsequent aggregation of globin might be enhanced via hydrophobic interaction of unfolded molecules. Copeland (1994) reported that elevated temperatures could greatly increase the rate of collisions between unfolded protein molecules. MetMb solution required a higher temperature for aggregation, indicating a less conformational change of metMb, in comparison with oxyMb over a wide range of temperatures.

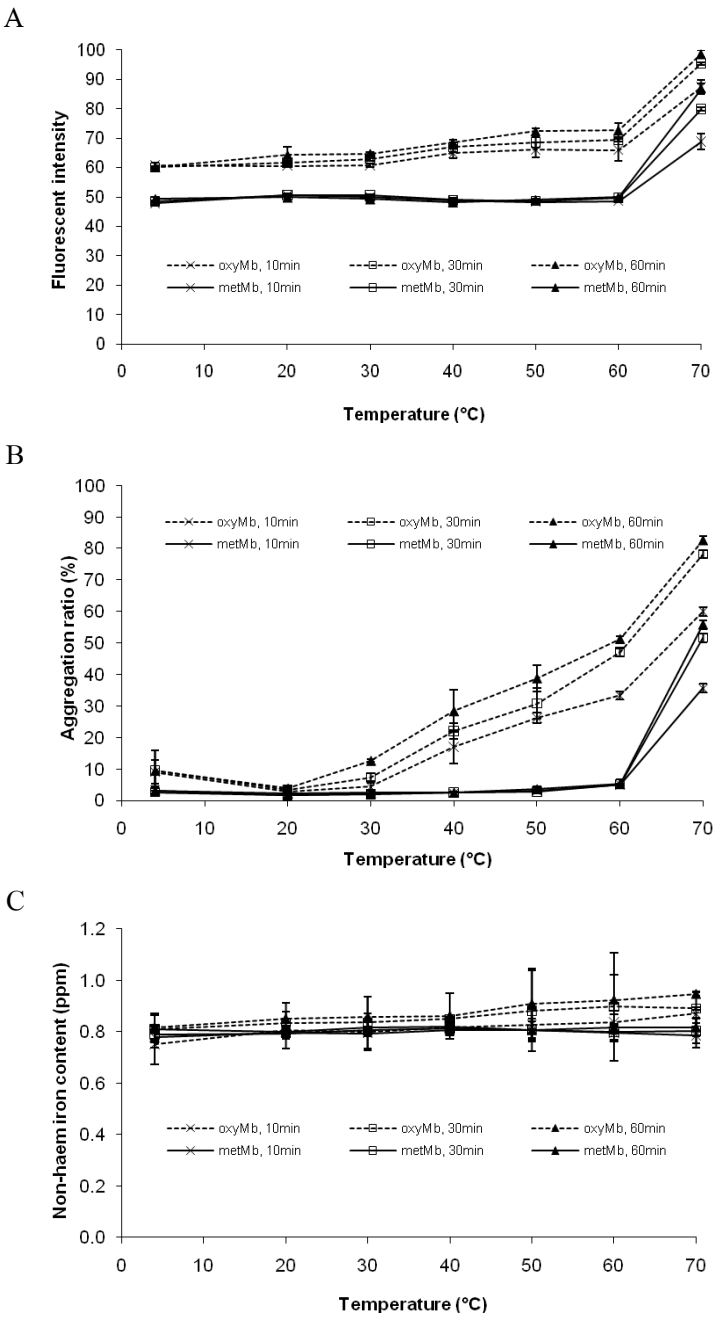


Figure 18. Effect of heating temperatures on tryptophan fluorescent intensity (A), aggregation ratio (B) and non-heme iron content (C) at pH 7 of oxyMb and metMb from Eastern little tuna dark muscle. Bars represent the standard deviation (n=3)

The release of non-heme iron content from oxyMb and metMb at varying temperature and time is shown in Figure 18C. When temperature increased up to 70ºC, no increase in non-heme iron content for metMb solution was observed ($P > 0.05$), whereas an increase was found in oxyMb solution with increasing temperature and time (0.75 to 0.95 ppm) ($P < 0.05$). Kristensen and Andersen (1997) reported a slight increase in free iron (0.46 to 0.50 ppm) from horse heart metMb after heating with temperature of 25 to 90ºC. The result suggested that increasing temperature up to 70ºC for 60 min had no effect on heme degradation and iron released of metMb solution, while conformational changes of apoMb in oxyMb solution might weaken a porphyrin ring, leading to iron released. Three important functions of the globin portion of Mb are to sequester heme, enhance coordination with the proximal histidine and inhibit oxidation of the iron atom (Liong *et al.*, 2001). Released iron might act as a pro-oxidant and was associated with the enhanced lipid oxidation. However, Kristensen and Andersen (1997) revealed that heated heme iron had a more significant effect on pro-oxidative activity than did heated free iron. Therefore, the suppression of conformational change should be required to maintain the heme structure and consecutively decrease pro-oxidative activity of Mb.

3.5 Conclusions

Mb isolated from dark muscle of Eastern little tuna with MW of 15,680 Da had different color and absorption spectra, depending on the forms, oxyMb and metMb. pH and heating could induce the destabilisation of Mb. Tuna Mb was prone to oxidation and denaturation at pH 3 and temperature above 60ºC. MetMb form was more stable than oxyMb. Processing of tuna muscle at mild conditions could lower the change of Mb, in which the color changes and pro-oxidative activity can be retarded. Therefore, the prime quality of tuna meat with high market value could be maintained during the handling or distribution.

CHAPTER 4

The effect of different atmospheric conditions on the changes in myoglobin and color of refrigerated Eastern little tuna (*Euthynnus affinis*) muscle

4.1 Abstract

Oxidation of myoglobin (Mb) is responsible for undesirable appearance and loss in acceptability of fish and fish products. The retardation of such a change by the modification of atmosphere would be a means to maintain the quality of fish during the refrigerated storage. The changes in oxyMb and metMb from dark muscle of Eastern little tuna (*Euthynnus affinis*) as affected by different atmospheric systems (closed system, opened system and flushed oxygen system) were determined. Saturated oxygen atmosphere more likely weakened heme-globin complex, especially as the exposure time increased. Autoxidation of oxy-form proceeded rapidly in the presence of oxygen with the concomitant formation of met-form. When the oxygen was excluded, oxidation of oxyMb was retarded. With flushed oxygen and increasing exposure time, the conformational changes of globin occurred, mainly associated with protein oxidation. Generally, oxyMb was more susceptible to oxidation and conformational change than did metMb. After keeping at 4ºC for 3 days, dark muscle of tuna fillet kept in vacuum packaging had a slight decrease in redness and it was still acceptable. The fillets stored in exposed air or packed in 100% O_2 atmosphere turned to be brown, most likely due to Mb oxidation. Thus, oxygen level of packaging atmosphere had a profound impact on Mb alteration, which was governed by the forms of Mb.

4.2 Introduction

Myoglobin (Mb) and Hemoglobin (Hb) are the most abundant heme compounds found in the animal muscle. Their physiological function is to carry and distribute oxygen to the different tissues, in which Hb is the main pigment in red blood cells and Mb is the main pigment

in the muscle cell. Mb presented in the post-mortem muscle is a major contributor to the color of meat and has a close relationship with lipid oxidation, depending on its redox states and concentration (Baron and Andersen, 2002; Sohn *et al.*, 2005).

Mb is made up of a single polypeptide chain, globin, and a prosthetic heme group, an iron (II) protoporphyrin-IX complex (Pegg and Shahidi, 1997). The converting process of ferrous Mb into ferric metMb is called autoxidation which is responsible for discoloration of meat and acceleration of lipid oxidation (Baron and Andersen, 2002; Sohn *et al.*, 2005). Haard (1992) reported that fish Mbs are at least 2.5 times more sensitive to autoxidation than mammalian counterpart. Thus, the stability of Mb is associated with different amino acid sequences and secondary structure of globin (Haard, 1992). Many factors have been known to contribute to autoxidation of Mb. Those include pH, temperature, oxygen and lipid oxidation products (Chaijan *et al.*, 2007; Chow *et al.*, 2004; Faustman *et al.*, 1999; Monahan *et al.*, 2005).

Autoxidation of Mb could produce metMb and superoxide anion which was converted to H_2O_2 by dismutation. Oxygen and oxygen consumption play the important role on autoxidation of deoxy- and oxyMb. Tissue oxygen consumption decreased with post-mortem time. Tang *el al.* (2005) found that bovine mitochondrial respiration in a closed system resulted in decreased oxygen partial pressure (pO_2) and enhanced the conversion of oxyMb to deoxyMb or metMb. Lee *el al.* (2003) reported that the grinding process for tuna (*Thunnus albacares*) patties is likely a cause of greater surface metMb formation in patties than in steaks because of the higher surface for air exposure. To minimize metMb formation in fresh beef, oxygen must either be totally excluded from the packaging environment or present at saturating levels (Faustman and Cassens, 1990).

Eastern little tuna (*Euthynnus affinis*) is a species available in the Gulf of Thailand and the Indian Ocean with the volume of 45,600 metric tons and a value of 37 million US dollars in 2007 which provides the high global economic value for canning and sashimi (Fisheries Foreign Affairs Division, 2007). The remarkably red color of tuna meat is caused by the high concentration of Mb (Lee *et al.*, 2003). The dark muscle of yellowfin tuna (*Neothunnus macropterus*) contained Mb ranging from 5.3 to 24.4 mg/g, while Hb was determined to range from 0.5 to 3.8 mg/g (Brown, 1962). To obtain the fish with the high acceptability in color and other qualities during the extended storage which may increase the commercial aspect of Eastern

little tuna, the suppression of Mb oxidation is required. To tackle the discoloration problem, the packaging condition or environment should be optimized. The objective of this investigation was to study the changes in Mb and color of fillets from Eastern little tuna under the different packaging conditions.

4.3 Materials and Methods

4.3.1 Chemicals

β-mercaptoethanol (βME), Triton X-100, pyridine and bathophenanthroline disulphonic acid were purchased from Sigma (St. Louis, MO, USA). Sodium dithionite was obtained from Riedel (Seeize, Germany). Sodium chloride, trichloroacetic acid, sodium nitrite and iron standard solution were procured from Merck (Damstadt, Germany).

4.3.2 Fish samples

Eastern little tuna (*E. affinis*) with the average weight of 0.5-0.55 kg were obtained from the dock in Songkhla province, Thailand. The fish were off-loaded 24 h after capture, placed in ice with a fish/ice ratio of 1:2 (w/w), transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within 1 h and immediately started for experiment.

4.3.3 Purification of Mb from Eastern little tuna

Five fish were filleted and the dark muscle was manually excised and collected. The composite sample was minced until it became uniform. Extraction and purification of Mb was performed according to the method of Thainsilakul *et al.* (2011). Dark mince of Eastern little tuna (100 g) was mixed with 300 ml of cold extracting medium (10 mM Tris-HCl, pH 8.0 containing 1 mM EDTA and 25 g/l Triton X-100). The mixture was homogenized for 1 min at a speed of 13,000 rpm using an IKA Labortechnik homogenizer (Selangor, Malaysia). After centrifugation at 9,600*g* for 10 min at 4ºC using an Avanti J-E centrifuge (Beckman Coulter, Palo Alto, CA, USA), the supernatant was filtered through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK). The filtrate referred to as “crude Mb extract” was subjected to

ammonium sulphate fractionation (65-100%) to remove other proteins. The precipitate obtained after centrifugation at 20,000*g* for 60 min was dissolved in a minimal volume of cold 5 mM Tris-HCl buffer, pH 8.5, which was referred to as "starting buffer". The mixture was then dialyzed against 10 volumes of the same buffer with 20 changes at 4ºC. To remove the contaminating protein, the dialysate was immediately applied onto a Sephadex G-75 column (2.6 × 70 cm; Amersham Bioscience, Uppsala, Sweden) which was previously equilibrated with the starting buffer. The separation was conducted using the starting buffer at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and measured at 280 and 540 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The fractions with the high absorbance at 540 nm were pooled and used as "tuna Mb".

4.3.3.1 Preparation of oxyMb and metMb

OxyMb and metMb were prepared according to the method of Tang *et al.* (2004) with some modifications. An aliquot (5 ml; 2 mg/ml) of tuna Mb was converted to oxyMb by the addition of 50 mg of sodium dithionite. To prepare metMb, 50 mg of potassium ferricyanide were added into 5 ml of the tuna Mb solution. The sodium dithionite and potassium ferricyanide were then removed by dialysis the sample against 10 volumes of cold 50 mM phosphate buffer, pH 7.0 with 20 changes. Protein concentrations of dialysates, representing oxyMb and metMb, were adjusted to 0.2 mg/ml.

4.3.3.2 Determination of Mb concentration

The concentration of Mb was determined by measuring the absorbance at 525 nm. The molar extinction coefficient of 7.6×10^{-3} and a molecular weight of 16,110 were used for calculation (Gomez-Basauri and Regenstein, 1992; Tang *et al.*, 2004). Mb content was expressed as mg/ml.

4.3.4 Effect of different atmospheric conditions on the changes of Eastern little tuna Mb

The solutions of oxyMb and metMb (1.7 ml, 0.2 mg/ml) were incubated in different atmospheric conditions for various times as per the methods of Tang *et al.* (2005) and Dosi *et al.* (2006) with some modifications. Those atmospheric conditions included: (1) "closed system", where the solutions were placed within the 1.7-ml microfuge tube (10 mm in diameter)

without any headspace, (2) “opened system”, in which the solutions were placed within the open-top microfuge tube which permits air to diffuse freely and (3) “flushed oxygen system”, where the solutions were placed in the test tube and bubbled with oxygen gas (99.5-100% purity; TIG Gas Agency, Hat Yai, Songkhla, Thailand) at a rate of 200 cm^3/min. All the procedures were carried out at 25ºC. The samples were collected every 30 min for totally 5 h. The changes in Mb were monitored.

4.3.4.1 Absorption spectra and Mb oxidation

Mb solutions were scanned from 350 to 750 nm using a UV-1800 spectrophotometer (Shimadzu, Japan) to determine the different forms of Mb (450 to 750 nm) and a loss of Soret band (350 to 450 nm) (Swatland, 1989). To determine the oxidation of Mb, the proportions of three Mb forms; deoxyMb, oxyMb and metMb were calculated following a modified Krzywicki’s equations (Tang *et al.*, 2004).

4.3.4.2 Tryptophan fluorescent intensity

To monitor the changes in protein structure, tryptophan fluorescent intensity of Mb solutions was measured using a RF-1501 fluorometer (Shimadzu, Kyoto, Japan) at an excitation wavelength of 280 nm and an emission wavelength of 325 nm according to the method of Chanthai *et al.* (1996).

4.3.4.3 Mb aggregation ratio

Mb solutions were filtered through a Millipore filter (pore size, 0.45 μm) (Millipore, Bedford, MA, USA). The filtrate was determined for the absorbance at 280 nm. The percentage of the decrease in absorbance relative to that of the initial sample (time=0) was calculated and used as Mb aggregation ratio (Chow *et al.*, 2004).

4.3.4.4 Non-heme iron content

Non-heme iron content of Mb solutions was determined to quantify the degree of heme degradation by the method of Schricker *et al.* (1982) with a slight modification. One ml of oxyMb and metMb was pipetted into a screw cap test tube and 50 μl of 0.39% sodium nitrite were added. Four ml of a mixture of 40% trichloroacetic acid and 6M HCl (ratio of 1: 1 (v/v), prepared freshly) were added. The tightly capped tubes were incubated at 65ºC for 22 h and then cooled at room temperature for 2 h. The supernatant (400 μl) was mixed with 2 ml of the non-heme iron color reagent. After vortexing and standing for 10 min, the absorbance was measured at 540 nm.

The non-heme iron content was calculated from iron standard curve. The iron standard solutions ($Fe(NO_3)$ in HNO_3), with the concentrations ranging from 0 to 2 ppm, were used.

4.3.5 Effect of different atmospheric packaging on the color and sensory property of Eastern little tuna fillet after refrigerated storage

The fillets of twenty Eastern little tuna (15 × 6 × 1 cm^3) having dark muscle were stored at 4ºC under the different atmospheric packaging conditions including (1) "air exposure" where tuna fillets (100-125 g) were placed into one layer on the plastic tray without any cover, (2) "vacuum" in which tuna fillets were packaged under vacuum packaging and (3) "extreme oxygen" where tuna fillets were packaged under modified atmosphere packaging (MAP) (100% O_2) using a gas/sample ratio of 2:1 (v/w). A nylon/PE bag (17.78 × 27.94 cm) with the selected gas permeability (O_2 transmission rate of 0.66 g m^{-2} day^{-1} at 23ºC, 101.33 kPa) and a Henko-vac type 1000 machine (Tecnovac, Italy) were used for vacuum packaging and MAP. After 3 days of storage at 4ºC, representing the condition for common distribution and retail for fillet, the samples were subjected to determinations. Three different lots of fish were used for the study.

4.3.5.1 Color measurement

Color of ordinary and dark muscle of tuna fillets were determined by measuring L^* (lightness), a^* (redness/greenness) and b^* (yellowness/blueness) values using a colorimeter (JP7100F, Juki Corp, Tokyo, Japan). The colorimeter was standardized by black and white tile and the samples were measured in standardized setting. Color differences, ΔE^*, were calculated by the following equation (Berns, 2000):

$$\Delta E^* = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}$$

where, ΔL^*, Δa^* and Δb^* represented the differences in the color parameters between the sample and the white standard.

4.3.5.2 Sensory evaluation

The appearance of tuna fillet samples was evaluated by thirty non-trained panelists using a 9-points hedonic scale ranging from dislike extremely (score = 1) to like extremely (score = 9) (Meilgaard *et al.*, 2006). The panelists were asked to evaluate for likeness of both dark and ordinary meat.

4.3.6 Statistical analysis

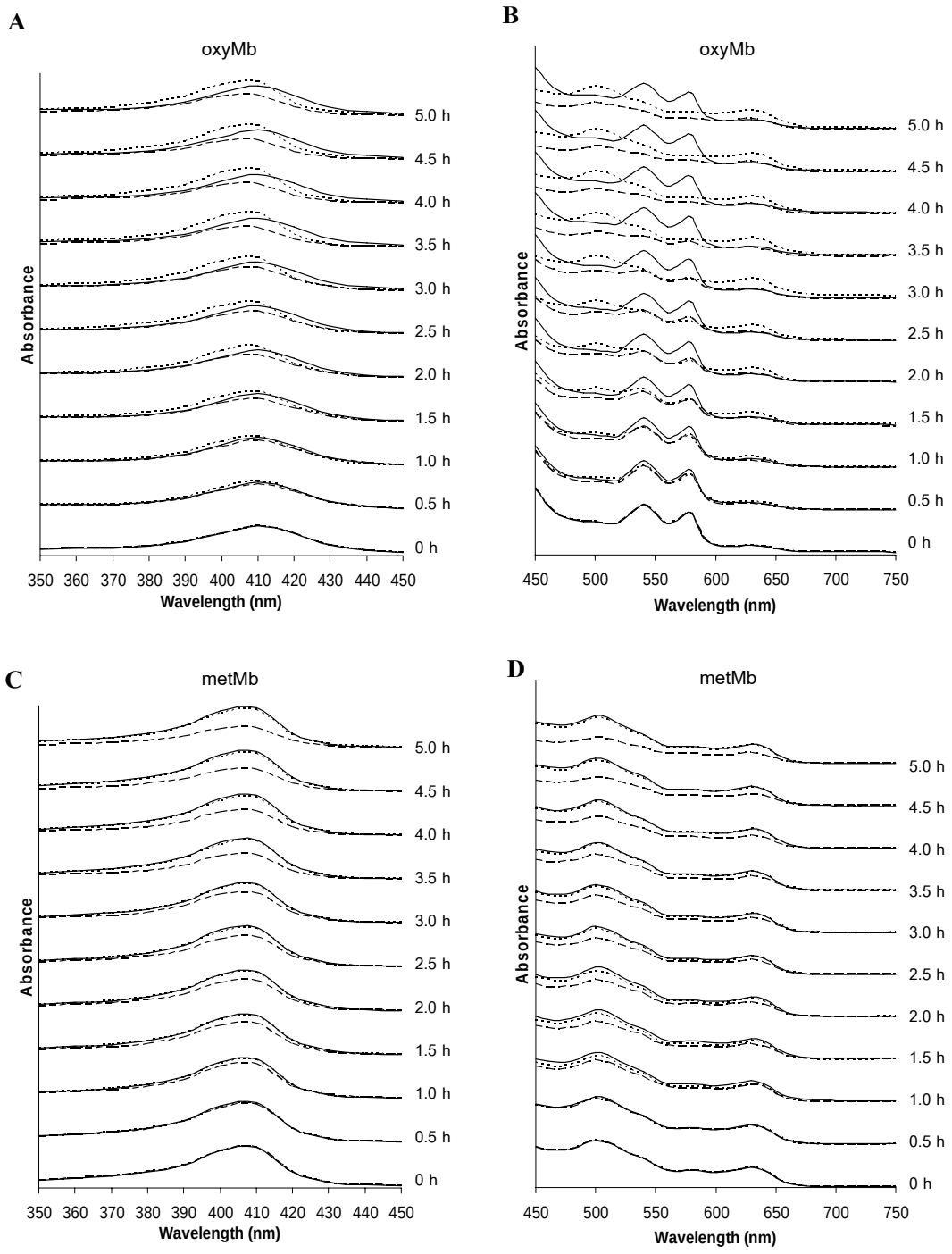
Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

4.4 Results and Discussion

4.4.1 Effect of atmospheric conditions on the changes of Eastern little tuna Mb

4.4.1.1 Changes in absorption spectra

The dark and ordinary muscle of Eastern little tuna contained Mb content of 11.77 and 2.34 mg/g sample, respectively. Prepared oxyMb solution had the highest proportions of oxyMb (69.94%), followed by metMb (21.62%) and deoxyMb (8.44%), respectively. For metMb solution, all forms of Mb were fully converted to metMb (100%). When the absorption spectra of both oxyMb and metMb stored under the different atmospheric conditions were determined, the intense peaks in a blue region (wavelength; 350-450 nm) corresponding to the Soret bands were found at 411 and 407 nm for oxyMb and metMb solutions, respectively (Figure 19A and 19C). Soret band results mainly from the interaction of the heme moiety with apoMb, hence it can be used to monitor the unfolding of hemoproteins (Chen and Chow, 2001). With increasing exposure time, oxyMb and metMb stored under the closed system exhibited a negligible change in Soret band, indicating the stability of heme-globin complex as well as the remaining redox state of both Mb forms in low oxygen atmosphere. For open atmosphere, no changes in Soret band were observed for metMb solution, while Soret band of oxyMb solution had the increase in peak area and shifted to the lower wavelength, especially as the storage time increased. This indicated that the oxidation of oxyMb took place when exposed to the air. The greater changes in Soret band were observed for both Mb solutions under the flushed oxygen system. When the exposure time increased, Soret band of oxyMb solution possessed the lower peak area and shifted to the lower wavelength, indicating a decrease in stability of heme-globin complex along with the enhanced oxidation of oxyMb at extreme oxygen tension. Additionally,



Closed system (—), Opened system (·····) and Flushed oxygen system (----)

Figure 19. Effect of different atmospheric conditions on the absorption spectra in a region of 350-450 and 450-750 nm of oxyMb (A and B) and metMb (C and D) solutions (pH 7.0 and 25ºC) from Eastern little tuna dark muscle as the function of exposure time

the lower Soret peak was noticeable for metMb solution under the flushed oxygen system when the exposure time increased. At high concentration of oxygen, a decrease in stability of heme-globin complex of both oxy- and metMb might be a result of oxidation of apoMb induced by oxygen. As a consequence, conformational change was obtained.

In a region of 450-750 nm, the peaks were found at wavelengths of 542 and 577 nm for oxyMb solution and 502 and 630 nm for metMb solution (Figure 19B and 19D). Typical spectra of oxyMb peak remained constant up to 5 h in the closed system. The typical absorption spectra of metMb were noticeable in oxyMb solution kept under the opened and flushed oxygen systems, as the exposure time increased. After 0.5 h of exposure time, the proportion of metMb formed increased to 37.00 and 27.21% (Table 5) ($P < 0.05$) for oxyMb solution stored under the opened system and flushed oxygen system, respectively. Nevertheless, no changes were noticeable for oxyMb solution kept under the closed system ($P > 0.05$), regardless of exposure time. The proportion of metMb indicated that a rapid autoxidation was more pronounced in oxyMb solution under the opened system, followed by that found in flushed oxygen system ($P < 0.05$). OxyMb stored under the closed system tended to have the oxygenated form as the dominant form, in which metMb formation of 29.47% was obtained after 5 h of exposure. The oxidation of Mb initiated by oxygen has been extensively reported (George and Stratmann, 1954; Wazawa *et al.*, 1992). Under air-saturated conditions (pO_2 = 20 kPa), almost all the Mb exists in the oxy-form with reversible oxygen binding. However, free oxygen rapidly oxidizes the ferrous heme to ferric heme, which can not bind oxygen. The reaction is shown as follows (Wazawa *et al.*, 1992):

$$Mb(II)(O_2) \longleftrightarrow Mb(II) + O_2$$

$$Mb(II) + O_2 \xrightarrow{H^+} MetMb(III) + O_2^-$$

Therefore, oxygen should be excluded from the surface of Mb solution without any headspace, leading to the less efficiency of oxygen to induce Mb autoxidation and to destabilize heme-globin complex. At an elevated pO_2, the concentration of oxyMb increases and can be further oxidized, resulting in the acceleration of metMb, superoxide radical and H_2O_2

Table 5. Proportion of metMb (%) of oxyMb and metMb solutions (pH 7.0 and 25ºC) from Eastern little tuna dark muscle under the different atmospheric conditions

Time (h)	oxyMb solution			metMb solution		
	Closed	Opened	Flushed O_2	Closed	Opened	Flushed O_2
0	$19.65\pm3.07^{gA*}$	19.65 ± 3.07^{iA}	19.65 ± 3.07^{kA}	101.40 ± 1.15^{aA}	101.40 ± 1.15^{aA}	101.40 ± 1.15^{aA}
0.5	20.62 ± 3.02^{fgC}	37.00 ± 2.12^{hA}	27.21 ± 3.74^{jB}	100.39 ± 2.05^{aA}	101.96 ± 0.79^{aA}	101.41 ± 0.71^{aA}
1.0	21.28 ± 2.35^{efgC}	49.41 ± 0.84^{gA}	32.42 ± 0.95^{iB}	100.14 ± 0.28^{aA}	100.57 ± 0.87^{aA}	100.12 ± 1.92^{aA}
1.5	22.62 ± 1.04^{defC}	60.30 ± 0.16^{fA}	38.53 ± 0.29^{hB}	99.68 ± 0.31^{aA}	99.99 ± 2.04^{aA}	100.46 ± 0.96^{aA}
2.0	23.91 ± 0.88^{cdeC}	71.54 ± 0.78^{eA}	45.00 ± 0.56^{gB}	100.39 ± 1.30^{aA}	100.64 ± 1.20^{aA}	100.93 ± 1.05^{aA}
2.5	25.14 ± 0.88^{bcdC}	82.23 ± 0.77^{dA}	52.74 ± 1.30^{fB}	101.00 ± 0.64^{aA}	101.48 ± 0.80^{aA}	100.85 ± 1.82^{aA}
3.0	26.62 ± 0.46^{abcC}	88.45 ± 0.77^{cA}	58.13 ± 1.78^{eB}	100.79 ± 0.73^{aA}	100.51 ± 1.08^{aA}	101.88 ± 0.81^{aA}
3.5	27.88 ± 0.83^{abC}	91.32 ± 0.77^{bA}	65.99 ± 1.93^{dB}	100.97 ± 1.32^{aA}	101.20 ± 1.22^{aA}	100.50 ± 2.20^{aA}
4.0	28.20 ± 0.47^{aC}	95.64 ± 0.56^{aA}	74.27 ± 1.06^{cB}	101.08 ± 4.18^{aA}	100.80 ± 0.89^{aA}	100.13 ± 1.73^{aA}
4.5	28.97 ± 0.46^{aC}	97.15 ± 0.13^{aA}	82.60 ± 1.30^{bB}	100.55 ± 1.14^{aA}	100.13 ± 1.92^{aA}	100.10 ± 1.77^{aA}
5.0	29.47 ± 0.32^{aC}	97.33 ± 1.93^{aA}	85.85 ± 1.60^{aB}	101.11 ± 0.50^{aA}	100.94 ± 1.29^{aA}	100.17 ± 1.94^{aA}

* Different superscripts in the same column indicate significant differences ($P < 0.05$). Different capital superscripts letters under the same Mb form within the same row indicate significant differences ($P < 0.05$).

formation (Brantley *et al.*, 1993; George and Stratmann, 1952; Weiss, 1982). A rapid oxidation for oxyMb exposed to high oxygen atmosphere suggested a powerful oxidizing power of oxygen molecule. However, oxyMb solution stored under the flushed oxygen atmosphere had a slower oxidation rate than that of the opened counterpart. This might be accompanied by a significant back reaction, in which oxyMb could be regenerated (Wazawa *et al.*, 1992).

MetMb solution stored under the varying conditions exhibited the typical spectra throughout the exposure of 5 h, although a slightly lower peak area was noticeable for metMb solution kept under the flushed oxygen system. No changes in the proportion of metMb indicated the stability of ferric state in all metMb solutions during the exposure of 5 h ($P > 0.05$). MetMb, a 6-coordinated iron with water molecule, which cannot attach oxygen, tends to be stable under any atmosphere (Pegg and Shahidi, 1997). As a consequence, no difference in oxidation state was observed for metMb kept under the varying atmospheric conditions.

4.4.1.2 Changes in tryptophan fluorescent intensity

Tryptophan fluorescent intensity of oxyMb and metMb solution from Eastern little tuna under the different atmospheric conditions is presented in Figure 20A. Tryptophan is involved in forming favourable and specific tertiary interactions of heme protein (Ivana *et al.*, 2000). An intrinsic fluorescent intensity of a tryptophan residue can be used to monitor the conformational changes of a tertiary structure (Chanthai *et al.*, 1996). With increasing exposure time, slight decreases in fluorescent intensity were observed for both oxyMb and metMb solutions stored under the closed system as well as the opened system ($P < 0.05$). For oxyMb solution, the opened system yielded the lower fluorescent intensity than that of the closed system. As the exposure time increased, a marked decrease in fluorescent intensity was noticeable for both Mb solutions stored under the flushed oxygen system. High concentration of oxygen can increase protein oxidation, resulting in the protein polymerization and protein fractionation (Lund *et al.*, 2007; Stadtman, 2001). Tryptophan, a heterocyclic amino acid, is susceptible to oxidation, yielding the various hydroxy derivatives (Stadtman, 2001). Kikugawa *et al.* (1991) reported a decrease in the amount of tryptophan residues after incubation with linoleic acid hydroperoxides. Therefore, a decrease in tryptophan fluorescent intensity for Mb solution under the flushed oxygen system might be a result of tryptophan oxidation. Apart from metMb, superoxide anion ($O_2^{\bullet -}$) and H_2O_2 are produced during oxidation of oxyMb. Those can act as a potent pro-oxidant

(Wazawa *et al.*, 1992). As a result, oxyMb might be prone to protein oxidation induced by those active species or oxidizing agents as evidenced by a sharp decrease in fluorescent intensity under the flushed oxygen system.

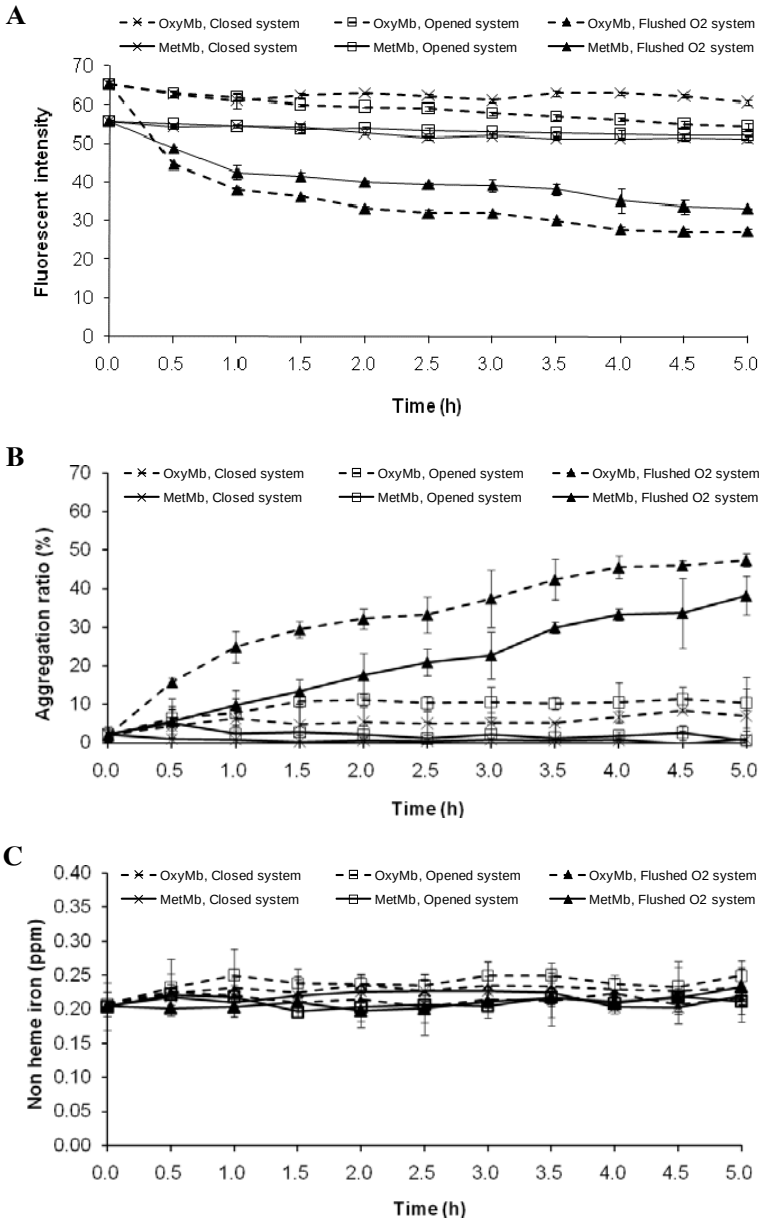


Figure 20. Effect of different atmospheric conditions on tryptophan fluorescent intensity (A), aggregation ratio (B) and non-heme iron content (C) of oxyMb and metMb solutions (pH 7.0 and 25ºC) from Eastern little tuna dark muscle as the function of exposure time. Bars represent the standard deviation (n=3)

4.4.1.3 Changes in aggregation ratio

Aggregation ratio of oxyMb and metMb solutions stored under different atmospheric conditions as a function of exposure time is presented in Figure 20B. No change in aggregation ratio was observed for oxyMb solution stored in the closed system ($P > 0.05$), while a slightly increased ratio was found in the opened system, especially with increasing exposure time ($P < 0.05$). Aggregation ratio remained constant in metMb solution stored under the closed and opened systems during 5 h of exposure ($P > 0.05$). These implied the stability of apoMb with the ferric form in air and under the low oxygen atmosphere. With flushed oxygen atmosphere, both oxyMb and metMb solutions showed a marked increase in aggregation ratio by 47.37 and 38.17% after 5 h of exposure, respectively ($P > 0.05$). The ratio increased continuously as the exposure time increased. A higher aggregation ratio under the flushed oxygen system was in accordance with a result of fluorescent intensity (Figure 20A). When proteins undergo oxidation, the obtained radicals can lead to the formation of cross-linked protein (Stadtman, 2001). These changes of protein conformation could be related with a weak heme-globin complex as evidenced by a decrease in Soret band of both Mb solutions under the flushed oxygen system.

4.4.1.4 Changes in non-heme iron content

Non-heme iron content of oxyMb and metMb solutions under the varying atmospheric conditions as a function of exposure time is presented in Figure 20C. When the exposure time increased, no changes in non-heme iron content were noticed for both Mb solutions in all systems used ($P > 0.05$). The result indicated that the oxygen concentrations had no significant effect on iron release from heme complex. Mb molecule contains one heme prosthetic group inserted into a hydrophobic cleft in the protein. Each heme residue contains one iron atom that is bonded to the four nitrogens of four pyrrole groups and to the imidazole group of histidine residue of globin (Pegg and Shahidi, 1997). The disruption of porphyrin ring probably occurred at harsh conditions such as very acidic pH and high temperature (Chaijan *et al.*, 2007), leading to the release of free iron called “non-heme iron”. Although a decrease in stability of heme-globin complex took place in flushed oxygen system as indicated by the change of Soret band, non-heme iron released was negligible. The result suggested that the detachment between heme and globin might occur without disruption of porphyrin ring. Grunwald and Richards (2006) reported that metMb promoted lipid oxidation more effectively than did ferrous Mb. This could be due to the

lower heme affinity of ferric Mb as compared to that of ferrous Mb (Grunwald and Richards, 2006). As a result, heme of metMb was more prone to be dissociated than was oxyMb. Since high oxygen concentration had an impact on the stability of Mb by affecting the oxidation state and protein conformation, the exclusion of oxygen should be required. However, the behaviour of *in vitro* Mb could be differed from that of post-mortem muscle, in which the Mb was localized in the compact system.

4.4.2 Effect of atmospheric packaging conditions on the color and sensory property of Eastern little tuna fillet during refrigerated storage

Color of Eastern little tuna fillet stored under different atmospheric packaging conditions including vacuum, opened air and MAP with 100% O_2 after 3 days of storage is shown in Figure 21. Immediately after filleting, meat color was quite deep purplish-red. As oxygen from the air contacted with the exposed meat surfaces, Mb was oxygenated and bloomed with the bright cherry red color (Figure 21A). After 3 days of chilled storage, fish fillet kept under the vacuum packaging turned to be pale for ordinary muscle and deep red for dark muscle (Figure 21B). The appearance of tuna fillets was poor in opened air and MAP with 100% O_2, in which the dark color in both ordinary and dark muscles was obtained (Figure 21C and 21D). Nevertheless, tuna fillets kept under the extreme oxygen atmosphere packaging had a glossy surface, whereas a more intense color and dried surface was detected in tuna fillets kept under exposed air due to a loss of moisture from the muscle.

As compared with the fresh tuna fillet (Table 6), the higher L^* and b^*-values were found in ordinary muscle of tuna fillet kept under vacuum packaging, while all packaging conditions obviously resulted in the higher L^* and b^*-values in dark muscle of tuna fillet. Thainsilakul *et al.* (2011) reported the higher L^* and b^*-values with a coincidental lower a^*-value of metmyoglobin from Eastern little tuna in comparison with oxy-form. This suggested the autoxidation of myoglobin likely occurred in dark muscle of tuna fillet under all packaging conditions. The ordinary muscle of tuna kept under exposed air had a^*-value similar to that of the fresh tuna. However, a higher ΔE^* was observed for the former. The a^* and ΔE^*-values were lower in fillets stored under vacuum and 100% O_2 MAP, compared with the fresh counterpart ($P < 0.05$). For dark muscle, changes of a^* and ΔE^*-value in tuna fillet kept under vacuum

packaging had the lowest changes, compared with the fresh tuna fillet. This result confirmed the efficiency of oxygen exclusion on myoglobin stability, in which the attractive redness of tuna dark muscle could be maintained. The opened air and modified atmosphere packaging with 100% O_2 more likely induced the oxidation of myoglobin, leading to metmyoglobin formation and discoloration of meat. Nevertheless, the maximum rate of metmyoglobin formation for beef was reported at pO_2 of 0.8-1.0 kPa, while autoxidation of myoglobin is minimized for meat held at high pO_2 (>4 kPa) (Robertson, 2006).

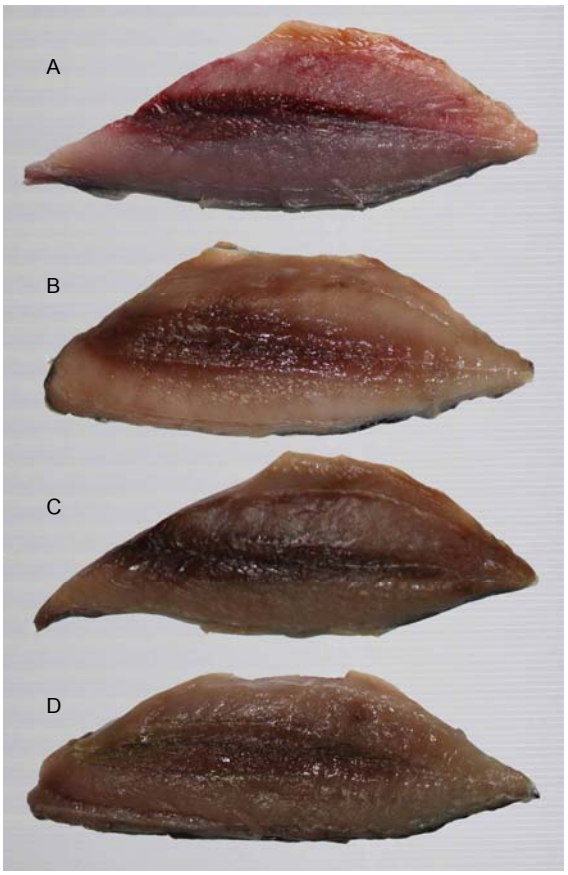


Figure 21. Fresh fillet of Eastern little tuna (A) and fillets stored under different atmospheric packaging: vacuum (B), exposed air (C) and modified atmosphere (100% O_2) (D) after storage at 4ºC for 3 days

Table 6. Color (a^* and ΔE^*-values) and appearance likeness of fresh fillets from Eastern little tuna and those stored under different packaging atmospheres at 4ºC for 3 days

Parameters			Fresh tuna	Storage condition (3 days)		
				Vacuum	Exposed air	100% O_2
Color	Ordinary muscle	a^*	4.87±0.86^{a†}	3.25±1.02^{b}	5.40±0.46^{a}	2.61±0.63^{b}
		ΔE^*	57.28±0.16^{b}	53.31±1.16^{c}	60.07±1.79^{a}	54.85±0.52^{c}
	Dark muscle	a^*	8.45±0.29^{a}	7.55±0.27^{b}	5.85±0.24^{c}	5.96±0.30^{c}
		ΔE^*	71.55±0.43^{a}	67.91±1.04^{b}	65.41±1.70^{c}	61.96±0.53^{d}
Appearance likeness††			8.8±0.4^{a}	7.6±0.9^{b}	5.1±1.7^{c}	3.8±1.9^{d}

† Different superscripts in the same row indicate significant differences ($P < 0.05$).

†† 9-points hedonic scale: dislike extremely (score = 1); like extremely (score = 9).

When appearance likeness of different samples with various atmospheric packaging was evaluated after 3 days of storage, the highest score was found in fresh tuna, followed by the sample kept under vacuum. For both fillets kept under the exposed air and oxygen rich atmosphere packaging, the lower score was associated with brown color development. Oxygen has sufficient oxidation potential to convert ferrous Mb to metMb, the undesirable color. Modified atmosphere retail packaging with a high level of oxygen (70-80%) was used for fresh meat to preserve the bright red color with sufficient shelf-life of meat (Eilert, 2005). Discoloration of tuna (*Thunnus albacares*) loins in MAP with 40%N_2/60%O_2 appeared after 14 days of storage at 2ºC whereas histamine formation was strongly inhibited under this condition (Emborg *et al.*, 2005). However, high concentration of oxygen most likely induced the

formation of metMb in Eastern little tuna fillet. In order to inhibit bacterial growth and oxidative reactions, MAP for fish and fishery products are normally contained 0-30% oxygen level (Sivertsvik *et al.*, 2002). This result was in accordance with Lund *et al.* (2007) who reported that high oxygen increased levels of oxidation in meat which can accelerate rancidity. Grunwald and Richards (2006) indicated that hemin loss rate was more critical in determining onset of lipid oxidation as compared to autoxidation rate. With extremely oxygen concentration, not only the heme autoxidation, but also the protein oxidation possibly occurred. This might also be associated with changes in color and lipid oxidation of tuna fillet containing a high amount of Mb.

4.5 Conclusions

A rapid autoxidation of Mb occurred when oxyMb was exposed to oxygen, whereas metMb was not affected by atmospheric conditions. High oxygen concentration had an impact on conformational change of globin, most likely due to the oxidation of proteins. No release of non-heme iron was observed, indicating integrity of heme domain even at extreme oxygen level. After keeping at 4ºC for 3 days, tuna fillets kept in exposed air and packed under 100% O_2 MAP had a poor appearance due to a dark color mediated by metMb formation. An acceptable tuna fillet was obtained when stored under vacuum packaging. The exclusion of oxygen could suppress the oxidation of Mb in both heme and globin portions.

CHAPTER 5

The effect of Fenton's reactants and aldehydes on the changes of myoglobin from Eastern little tuna (*Euthynnus affinis*) dark muscle

5.1 Abstract

The influences of Fenton's reactants (H_2O_2 and $FeCl_2$) and aldehydes (hexanal and hexenal) on changes of myoglobin (Mb) including oxyMb and metMb from Eastern little tuna (*Euthynnus affinis*) dark muscle were studied. In the presence of H_2O_2, both oxy- and metMb were rapidly oxidized into ferrylMb based on spectra patterns. In the presence of Fe^{2+} and/or H_2O_2, the changes in fluorescent intensity of Mb were noticeable, but there were no changes in aggregation ratio. Release of non-heme iron from Mb was mainly governed by H_2O_2. When aldehydes were incorporated, the oxidation of oxyMb and conformational changes of globin were more pronounced. No release of non-heme iron was noticeable, suggesting the stability of heme moiety toward aldehydes. Hexenal had a great impact on cross-linking of oxyMb and metMb via covalent modification. Alteration of Mb redox state might be enhanced by conformational changes of globin induced by both Fenton's reactants and aldehydes.

5.2 Introduction

Myoglobin (Mb), a predominant pigment protein found in dark fleshed-fish species, has been suggested for its relationship with lipid oxidation (O'Grady *et al.*, 2001; Richards *et al.*, 2005). Lipid oxidation is a major cause of deterioration of food and food products, especially those containing high content of unsaturated fatty acids. Oxidation of Mb is associated with the accelerated lipid oxidation (O'Grady *et al.*, 2001). The role of hemin dissociation in the ability of different heme proteins to promote lipid oxidation processes was reported by Richards *et al.* (2005).

DeoxyMb and oxyMb are in the ferrous state (Fe^{2+}), whereas metMb is in the ferric state (Fe^{3+}). Autoxidation of oxyMb results in formation of metMb and superoxide ($^{\bullet}OOH/ O_2^{\bullet-}$), which rapidly dismutate to H_2O_2 and O_2 (Chan *et al.*, 1997). The interaction of H_2O_2 with metMb led very rapidly to generation of active species, perferrylMb ($^{\bullet}$MbFe(IV)=O) and ferrylMb species (MbFe(IV)=O), which could initiate lipid peroxidation (Baron *et al.*, 2000; Chan *et al.*, 1997). The ferrous Mb species, deoxyMb and oxyMb, can likewise react with H_2O_2, resulting in the formation of ferrylMb by direct two-electron oxidation (Davies, 1991). Additionally, $O_2^{\bullet-}$ can also reduce dissociated hemin to heme which further reacts with H_2O_2 to form either hydroxyl radical or a non-protein bound ferryl-heme (Robinson *et al.*, 2009). These products are able to abstract a hydrogen atom from a polyunsaturated fatty acid and initiates lipid oxidation. Chan *et al.* (1997) revealed that H_2O_2, presumably generated during oxyMb oxidation, played an important role in mediating oxyMb and lipid oxidation in liposome system. The oxidation of phosphatidylcholine by ferrylMb was found to be approximately seven-fold greater than that observed for native Mb (Vuletich *et al.*, 2000). The potential of ferrylMb to oxidize lipids is dependent on H_2O_2, lipid hydroperoxide production, the concentration of reducing agents and their compartmentalization in the muscle cells (Baron and Andersen, 2002).

Lipid oxidation could generate a variety of secondary products, predominantly *n*-alkanals, *trans*-2-alkenals, 4-hydroxy-*trans*-2-alkenals and malondialdehyde (Faustman *et al.*, 1999). The aldehyde products are more water-soluble than their parent compounds and could potentially interact with Mb (Faustman *et al.*, 1999; Lynch and Faustman, 2000). Hexanal, hexenal and 4-hydroxynonenal were reported to enhance the oxidation of tuna oxyMb (Lee *et al.*, 2003a). Porcine metMb formation was greater in the presence of 4-hydroxynonenal (Lee *et al.*, 2003b). Lynch and Faustman (2000) also determined the effect of aldehyde lipid oxidation products on oxyMb oxidation, metMb reduction and the catalytic activity of metMb as a lipid pro-oxidant *in vitro*. Aldehydic lipid oxidation products covalently attached to Mb and subsequently cause structural alterations, which would make the protein more susceptible to oxidation (Faustman *et al.*, 1999).

The process of ferrous Mb converting into ferric metMb is responsible for discoloration of meat and acceleration of lipid oxidation (Baron and Andersen, 2002; Lee *et al.*, 2003a). However, less information regarding the influence of the oxidation products of Mb and

lipid on stability of fish Mb has been reported. A better understanding of this subject would provide promising approaches for inhibition of lipid oxidation as well as improving overall quality of fish or meat. Eastern little tuna (*Euthynnus affinis*) is a species available in the Gulf of Thailand and the Indian Ocean. It provides the high global economic value for canning and sashimi (Fisheries Foreign Affairs Division, 2007). This species contains red muscle with a high amount of Mb, which may undergo oxidation with ease, especially in the presence of pro-oxidative compounds, such as Fenton's reactants and aldehydic lipid oxidation products. Therefore, the objective of this investigation was to study the stability of Mb from Eastern little tuna dark muscle as influenced by Fenton's reactants and selected aldehydes.

5.3 Materials and Methods

5.3.1 Chemicals

β-mercaptoethanol (βME), Triton X-100, pyridine, hexanal, trans-2-Hexen-1-al, low range protein markers and bathophenanthroline disulfonic acid were purchased from Sigma (St. Louis, MO, USA). Sodium dithionite and ferrous chloride were obtained from Riedel (Seeize, Germany). Hydrogen peroxide, trichloroacetic acid, sodium nitrite and iron standard solution were procured from Merck (Damstadt, Germany).

5.3.2 Fish collection and preparation

Twenty Eastern little tuna (*E. affinis*) with the average weight of 0.5-0.55 kg were obtained from the dock in Songkhla province, Thailand. The fish were off-loaded 24 h after capture, placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within 1 h. Upon arrival, fish were washed with cold water (5ºC), beheaded and eviscerated. Thereafter, the fish were filleted and the dark muscle was manually excised and collected within the same batch. The composite sample was minced until the uniformity was obtained.

5.3.3 Purification of Mb from Eastern little tuna

Extraction and purification of Mb was performed according to the method of Thiansilakul *et al.* (2011). Eastern little tuna mince (100 g) was mixed with 300 ml of cold extracting medium (10 mM Tris-HCl, pH 8.0 containing 1 mM EDTA and 25 g/l Triton X-100). The mixture was homogenized for 1 min at a speed of 13,000 rpm using an IKA Labortechnik homogenizer (Selangor, Malaysia). After centrifugation at 9,600*g* for 10 min at 4ºC using an Avanti J-E centrifuge (Beckman Coulter, Palo Alto, CA, USA), the supernatant was filtered through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK). The filtrate referred to as "crude Mb extract" was subjected to ammonium sulfate fractionation (65-100% saturation). The precipitate obtained after centrifugation at 20,000*g* for 60 min was dissolved in a minimal volume of cold 5 mM Tris-HCl buffer, pH 8.5, which was referred to as "starting buffer". The mixture was then dialyzed against 10 volumes of the same buffer with 20 changes at 4ºC. The dialysate was immediately applied onto a Sephadex G-75 column (2.6 × 70 cm; Amersham Bioscience, Uppsala, Sweden) which was previously equilibrated with the starting buffer. The separation was conducted using the starting buffer at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and measured at 280 and 540 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The fractions with the high absorbance at 540 nm were pooled and used as "tuna Mb".

5.3.3.1 Preparation of oxyMb and metMb

OxyMb and metMb were prepared according to the method of Tang *et al.* (2004) with some modifications. An aliquot (5 ml; 0.25 mM) of tuna Mb was converted to oxyMb by the addition of 0.1 g of sodium dithionite. MetMb was prepared by adding 0.1 g of potassium ferricyanide to 5 ml of the tuna Mb solution. The sodium dithionite and potassium ferricyanide were then removed by dialysis the sample against 10 volumes of cold 50 mM phosphate buffer, pH 7.0 with 20 changes of dialysis buffer. The concentrations of oxyMb and metMb were adjusted to 0.2 mM.

5.3.3.2 Determination of Mb concentration

The concentration of Mb (mM) was determined by measuring the absorbance at 525 nm. The molar extinction coefficient of 7.6×10^{-3} was used for calculation (Tang *et al.*, 2004).

5.3.4 Effect of Fenton's reactants on the changes of Eastern little tuna Mb

The changes in oxyMb and metMb as affected by Fenton's reactants were monitored according to the method of O'Grady *et al.* (2001) with a slight modification. In a final volume of 4 ml, the solutions of oxyMb and metMb (0.2 mM) were incubated with $FeCl_2$ (0.2 mM) and/or H_2O_2 (0.1 mM). The different procedures for addition of $FeCl_2$ and/or H_2O_2 into Mb solutions were used. Those included Mb+$FeCl_2$ (MF), Mb+H_2O_2 (MH), Mb+$FeCl_2$+H_2O_2 (MFH), Mb+H_2O_2+$FeCl_2$ (MHF), Mb+the mixture of $FeCl_2$ and H_2O_2 previously mixed for 1, 5 and 10 min (MM1, MM5 and MM10). After the thorough mixing, all samples were stored at 4ºC for 1 h and analyzed for the changes of Mb.

5.3.5 Effect of aldehydes on the changes of Eastern little tuna Mb

To study the effect of hexanal and hexenal on the changes of oxyMb and metMb, the solutions of oxyMb and metMb (0.15 mM) were added with hexanal or hexenal (1 mM) at a ratio of 1:1 to obtain a final concentration of 0.5 mM aldehyde. Control was aldehyde-free and was prepared in the same manner, except an equivalent volume of ethanol was used instead. The treated samples were collected at 0, 1, 2, 4, 6 and 8 days of incubation at 4ºC for analyzes. To determine the cross-linking effect of aldehydes, the mixture containing hexanal and hexenal at a final concentration of 12.5 mM was also prepared and incubated at 4ºC for 1 day prior to SDS-PAGE analysis.

5.3.6 Analyzes heme protein degradation and Mb oxidation

Heme protein degradation was determined by the changes in absorption spectra and Soret band (Swatland, 1989). The proportions of three Mb forms; deoxyMb, oxyMb and metMb were calculated following a modified Krzywicki's equations (Tang *et al.*, 2004). To determine the oxidation of Mb by the effect of Fenton's reactants, the ratio of absorbance at 580 and 525 nm (A_{580}/A_{525}) was calculated (Lee *et al.*, 2003a). A high A_{580}/A_{525} value indicates a high proportion of oxyMb.

5.3.6.1 Tryptophan fluorescent intensity

Tryptophan fluorescent intensity of treated Mb solutions was measured using a RF-1501 fluorometer (Shimadzu, Kyoto, Japan) at an excitation wavelength of 280 nm and an emission wavelength of 325 nm according to the method of Chanthai *et al.* (1996).

5.3.6.2 Mb aggregation ratio

Mb solutions were filtered through a Millipore filter (pore size, 0.45 μm) (Millipore, Bedford, MA, USA). The filtrate was determined for the protein content. The percentage of the decrease in protein content relative to that of the initial sample (time=0) was calculated as Mb aggregation ratio (Chow *et al.*, 2004).

5.3.6.3 Non-heme iron content

Non-heme iron content of Mb solutions was determined according to the method of Schricker *et al.* (1982) with a slight modification. One ml of oxyMb and metMb solutions was pipetted into a screw cap test tube and 50 μl of 0.39% (w/v) sodium nitrite were added. Four ml of a mixture of 40% trichloroacetic acid and 6 M HCl (ratio of 1: 1 (v/v), prepared freshly) were added. The tightly capped tubes were incubated at 65ºC for 22 h and then cooled at room temperature for 2 h. The supernatant (400 μl) was mixed with 2 ml of the non-heme iron color reagent, a mixture of bathophenanthroline disulfonate reagent (0.162 g of bathophenanthroline disulfonic acid + 100 ml of double-deionized water + 2 ml of thioglycolic acid), double-deionized water and saturated sodium acetate solution at a ratio of 1:20:20 (v/v/v), prepared freshly. After vortexing and standing for 10 min, the absorbance was measured at 540 nm. The non-heme iron content was calculated from iron standard curve. The iron standard solutions ($Fe(NO_3)$ in HNO_3), with the concentrations ranging from 0 to 2 ppm, were used.

5.3.6.4 SDS-polyacrylamide gel electrophoresis

To determine the protein pattern of Mb, the treated Mb solutions were subjected to SDS-PAGE according to the method of Laemmli (1970). Samples with reducing and non-reducing conditions were loaded onto polyacrylamide gels comprising 4% stacking gel and 17.5% running gel and subjected to electrophoresis using a Mini PROTEAN II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). The low-molecular-weight-protein standards including bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor

(20 kDa), α-lactalbumin (14.2 kDa) and aprotinin (6.5 kDa) were used for molecular weight estimation.

5.3.7 Statistical analysis

Experiments were run in triplicate using three different batches of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

5.4. Results and Discussion

5.4.1 Effect of Fenton's reactants on the changes of Eastern little tuna Mb

5.4.1.1 Changes in absorption spectra

OxyMb solution contained oxyMb as the predominant form (68.91%), followed by metMb (23.64%) and deoxyMb (7.45%), respectively. For metMb solution, a complete metMb formation (100%) was found. The strong absorption of Mb was located in a wavelength region of 350-450 nm or Soret band, in which the intense peaks at 413 and 406 nm were observed for the control oxyMb and metMb, respectively (Figure 22). The typical spectrum in a region of 450-750 nm representing the redox state of Mb had the peaks at wavelengths of 542 and 577 nm for oxyMb and of 502 and 630 nm for metMb.

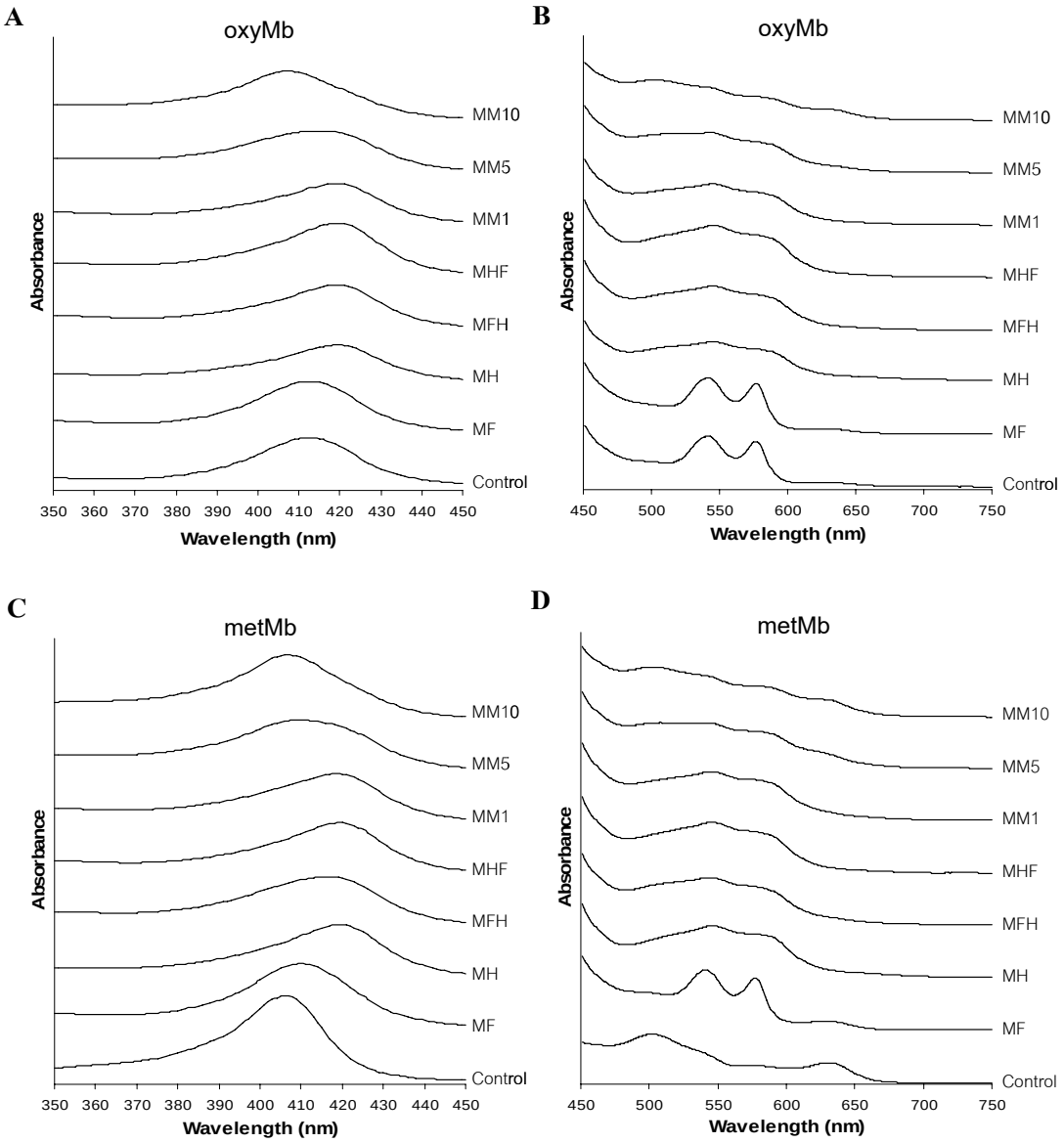


Figure 22. Effect of Fe^{2+} and/or H_2O_2 on the absorption spectra in a region of 350-450 and 450-750 nm of oxyMb (A and B) and metMb (C and D) solutions from Eastern little tuna dark muscle

When Fe^{2+} and/or H_2O_2 were added, Mb redox state was altered as evidenced by the changes in absorption characteristics of oxyMb and metMb solutions. Absorbance spectra of MF for oxyMb and metMb solution were similar to that of the control oxyMb solution, suggesting the reducing capability of Fe^{2+}. The highest oxyMb content was obtained in the control and MF of oxyMb solution and MF of metMb solution ($P < 0.05$) (Table 7). In the presence of H_2O_2 with or without Fe^{2+} added, the great changes in absorption spectra were observed in both oxyMb and metMb solutions. MH, MFH and MHF from oxyMb and metMb solutions presenting a Soret peak shifted to a higher wavelength (~419 nm). Additionally, they had the increased absorbance in the region of 500-600 nm along with the unsharpened peak at wavelength of ~545 nm. The similar spectra patterns were evidenced for ferrylMb of sperm whale and horse heart (Cooper *et al.*, 2005; Romero *et al.*, 1992). Prasad *et al.* (1989) reported the similar wavelength of absorption peaks between oxyMb and ferrylMb from horse heart by which the latter had the drastic decrease in peak height. Therefore, both oxyMb and metMb could be rapidly oxidized by H_2O_2, leading to the formation of ferrylMb via one or two-electron oxidation (Davies, 1991; O'Grady *et al.*, 2001). For both oxyMb and metMb solutions, MM1 more likely exhibited the typical spectrum of ferrylMb. Conversely, MM5 and MM10 tended to possess the different absorption spectra. Increasing time for Fenton's reaction (Fe^{2+} + H_2O_2) might decrease the H_2O_2 content and concurrently increased the amount of hydroxyl radicals which exhibited the pro-oxidative effect on Mb. During the oxidation of oxyMb solutions as accelerated by H_2O_2 or hydroxyl radical, the decreases in oxyMb contents were noticeable and the lowest content was obtained in MM10 for both oxyMb and metMb solutions ($P < 0.05$) (Table 7). In the presence of H_2O_2 or Fenton's reactants, A580/A525, which indicated the proportion of oxyMb form, was greater in MH, compared with the control for metMb solution ($P < 0.05$). This possibly resulted from a presence of ferrylMb, an oxygen containing Mb (MbFe(IV)=O), which had a typical spectrum related to that of oxyMb form (Prasad *et al.*, 1989). O'Grady *et al.* (2001) revealed that the oxidation of oxyMb in the presence of both Fenton's reactants may not involve hydroxyl radical production but proceeds via a direct interaction between H_2O_2 and oxyMb. Thus, H_2O_2 was a necessary reactant in the conversion of oxyMb or metMb to a hypervalent, ferrylMb, which is known to be an effective pro-oxidant (Baron and Andersen, 2002; O'Grady *et al.*, 2001).

Table 7. A_{580}/A_{525} value of oxyMb and metMb solutions from Eastern little tuna dark muscle as affected by Fe^{2+} and/or H_2O_2

Treatments	A_{580}/A_{525} †	
	oxyMb	metMb
Control	$1.24\pm0.08^{aA*}$	0.48 ± 0.00^{gB}
MF	1.22 ± 0.07^{aA}	1.13 ± 0.06^{aA}
MH	0.89 ± 0.01^{bB}	0.95 ± 0.00^{bA}
MFH	0.89 ± 0.01^{bA}	0.87 ± 0.01^{deA}
MHF	0.91 ± 0.01^{bA}	0.91 ± 0.01^{cdA}
MM1	0.87 ± 0.01^{bB}	0.91 ± 0.00^{bcA}
MM5	0.79 ± 0.01^{cB}	0.83 ± 0.00^{eA}
MM10	0.67 ± 0.00^{dB}	0.71 ± 0.00^{fA}

† A high A_{580}/A_{525} value indicates a high proportion of oxyMb

* Different superscripts in the same column indicate significant differences ($P < 0.05$). Different capital superscripts in the same row indicate significant differences ($P < 0.05$).

5.4.1.2 Changes in tryptophan fluorescent intensity

Changes in tryptophan fluorescent intensity of oxyMb and metMb solutions from Eastern little tuna as affected by Fe^{2+} and/or H_2O_2 are depicted in Figure 23A. The highest tryptophan fluorescent intensity was found in MH of both Mb solutions ($P < 0.05$). H_2O_2 was not only capable of oxidizing oxyMb and metMb into ferrylMb, but also enhanced conformational changes of apoMb as evidenced by a marked increase in fluorescent intensity. DeGray *et al.* (1997) reported that the reaction between H_2O_2 and recombinant sperm whale Mb resulted in the formation of globin-centered radicals, especially at tyrosine and tryptophan residues. Decreases in fluorescent intensity were observed in MF, MFH, MHF, MM1, MM5 and MM10 of both Mb solutions, as compared with the corresponding controls ($P < 0.05$). Heavy metal salt such as $FeCl_2$ added into Mb solutions might act as the protein denaturant by disruption of electrostatic interaction, resulting in an irreversible conformational change or denaturation of protein (Bontidean *et al.*, 1998). Hydroxyl radicals produced from Fenton's reaction could oxidize the

proteins, contributing to either protein cross-linking or fragmentation (Gajewski and Dizdaroglu, 1990; Uchida *et al.*, 1990). Moreover, the modified protein might bury tryptophan or aromatic domain inside, as evidenced by the decrease in fluorescent intensity. However, slight differences in fluorescent intensity among MFH, MHF, MM1, MM5 and MM10 of both Mb solutions could be observed ($P < 0.05$). This might be related with the different pro-oxidant effect of hydroxyl radicals towards apoMb. When comparing the impact of Fenton's reaction time on fluorescent intensity, it was found that reaction time had no influence on fluorescent intensity of metMb. Nevertheless, the longer reaction caused the gradual decrease in fluorescent intensity of oxyMb solution. Furthermore, the order of addition of reactant (H_2O_2 and $FeCl_2$) only had the effect on fluorescent intensity of oxyMb ($P < 0.05$). Thus, the globin configuration was affected by Fenton's reactants, but the degree of changes depended on the form of Mb.

5.4.1.3 Changes in aggregation ratio

Aggregation ratio of oxyMb and metMb from Eastern little tuna in the presence of Fe^{2+} and/or H_2O_2 is shown in Figure 23B. Following 1 h of incubation at 4ºC, aggregation ratio of the control oxyMb and metMb were 19.84 and 14.00%, respectively. For both Mb solutions, no differences in aggregation ratio were observed for MH and its control ($P > 0.05$). The lower ratios were noticeable in the presence of Fe^{2+}, regardless of H_2O_2 added ($P < 0.05$). Decrease in stabilization of the native structure of protein is presumed to be the key step for protein aggregation (Chi *et al.*, 2003). H_2O_2 showed the insignificant effect on Mb aggregation, while Fe^{2+} and Fenton's reactants could suppress the aggregation of Mb. OxyMb and metMb solutions which had the low tryptophan fluorescent intensity (Figure 23A), might have hydrophilic amino residues existing at exterior portion along with charge shielding from iron salt ($FeCl_2$). Therefore, aggregation of globin enhanced via hydrophobic interaction could be reduced (Chi *et al.*, 2003). The control and MH of oxyMb solution exhibited the higher aggregation ratios than those of metMb solution ($P < 0.05$). This suggested a more conformational change of oxyMb in comparison with metMb regardless of H_2O_2 incorporation. However, no differences in aggregation ratio were observed between oxyMb and metMb solutions when Fe^{2+} was incorporated ($P > 0.05$). No difference in protein patterns among all samples was noticeable (data not shown), indicating negligible change in the molecular weight of Mb as influenced by Fenton's reactants.

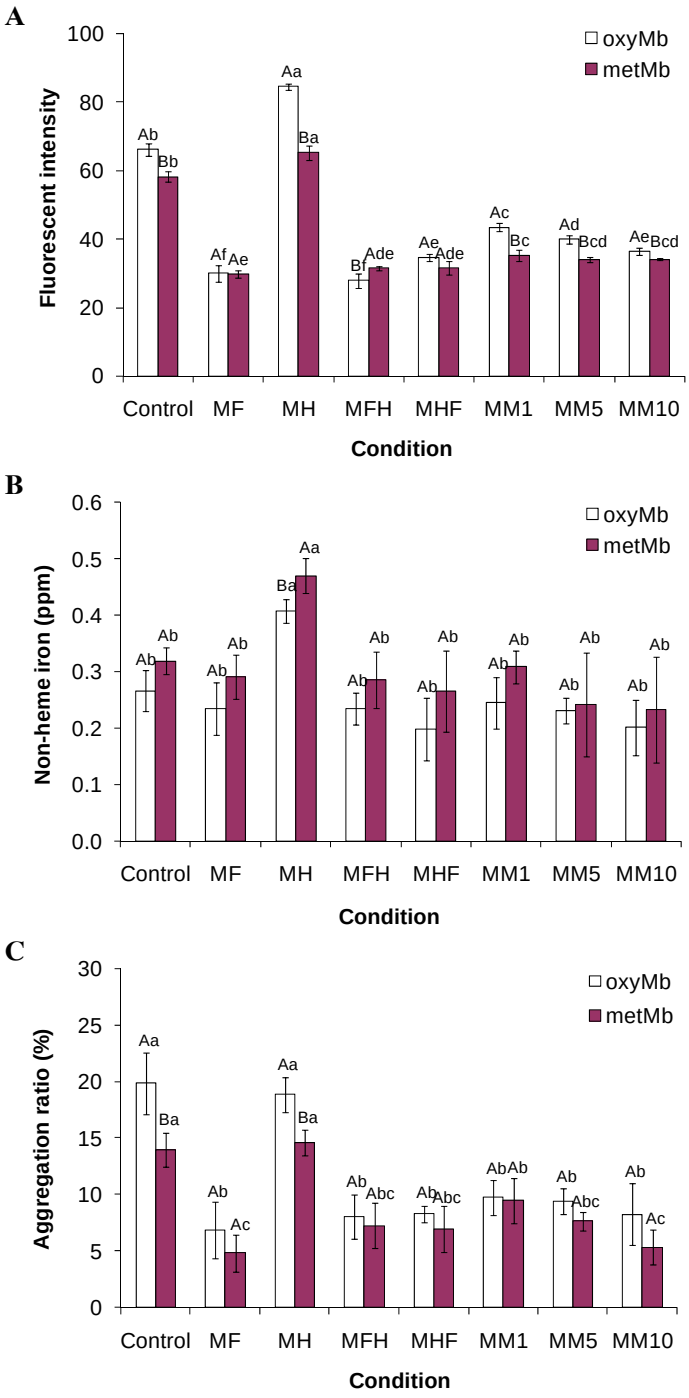


Figure 23. Effect of Fe^{2+} and/or H_2O_2 on tryptophan fluorescent intensity (A), aggregation ratio (B) and non-heme iron content (C) of oxyMb and metMb solutions from Eastern little tuna dark muscle. Bars represent the standard deviation (n=3). Different letters within the same Mb form indicate significant differences ($P < 0.05$). Different capital letters within the same treatment indicate significant differences ($P < 0.05$)

5.4.1.4 Changes in non-heme iron content

The release of non-heme iron content from oxyMb and metMb solutions as affected by Fe^{2+} and/or H_2O_2 is shown in Figure 23C. Non-heme iron content increased, when H_2O_2 was added into oxyMb and metMb solution ($P < 0.05$). Normally, heme group is surrounded in a hydrophobic pocket-like structure of globin, in which iron is occupied four sites with nitrogen of porphyrin ring and the other one site with histidine residue of globin (Livingston and Brown, 1981). H_2O_2 with oxidizing power might induce the change of Mb redox state as well as structure of globin, thereby enhancing iron released from porphyrin ring. In the presence of Fe^{2+} with or without H_2O_2 added, no changes in iron released from both Mb solutions were observed, compared with the control Mb ($P > 0.05$). It suggested that porphyrin ring of heme in hydrophobic pocket of Mb was probably stable in the presence of Fe^{2+} or Fenton's reactants. For the same treatment, no difference in non-heme iron content was noticeable between oxyMb and metMb except for MH that H_2O_2 might cause the release of free iron from metMb at a higher extent.

5.4.2 Effect of aldehyde on the changes of Eastern little tuna Mb

5.4.2.1 Changes in absorption spectra

The effect of hexanal and hexenal on changes in absorption spectra of oxyMb and metMb solutions from Eastern little tuna is presented in Figure 24. For oxyMb solution, as the incubation time increased up to 8 days, the peak of Soret bands was slightly increased in intensity and shifted to a lower wavelength. Concurrently, the typical spectra of oxyMb in a region of 450-750 nm gradually disappeared. These implied the alteration of oxyMb into an oxidized form induced by aldehyde, especially with the sufficient incubation time. After incubation for 1 day, the proportion of metMb form in the control and oxyMb solution incorporated with hexanal and hexenal increased to 31.03, 34.48 and 34.90%, respectively ($P < 0.05$). With increasing incubation time up to 8 days, the highest formation of metMb was found in oxyMb solution added with hexenal (76.47%), followed by hexanal (71.07%) and the control (68.73%), respectively ($P < 0.05$). The result suggested that the oxidation of oxyMb was induced by aldehydes, in which hexenal had a greater impact than its saturated counterpart, hexanal. Faustman *et al.* (1999) and Lynch and Faustman (2000) reported that metMb formation was

greater in the presence of α-β-unsaturated aldehydes than their saturated counterparts having equivalent carbon chain length. A negligible change in the absorption spectra was observed for metMb solutions in both 350-450 nm and 450-750 nm regions, regardless of incubation time, indicating that aldehydes had no effect on the stability of metMb.

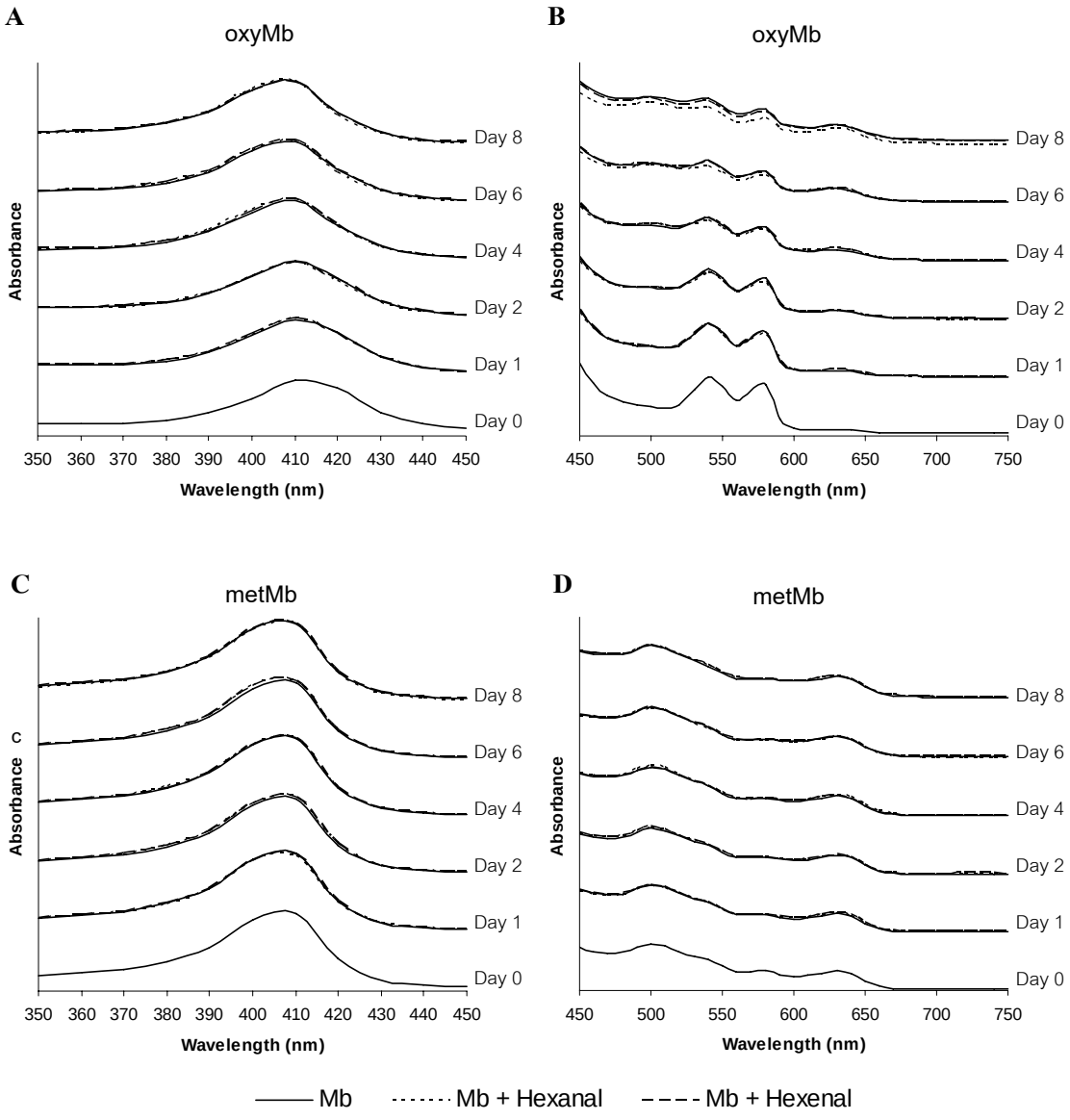


Figure 24. Effect of hexanal and hexenal on the absorption spectra in a region of 350-450 and 450-750 nm of oxyMb (A and B) and metMb (C and D) solutions from Eastern little tuna dark muscle as the function of incubation time

5.4.2.2 Changes in tryptophan fluorescent intensity

Changes in tryptophan fluorescent intensity of oxyMb and metMb solutions in the presence of hexanal and hexenal during the incubation up to 8 days are presented in Figure 25A. Within the first 1 day of incubation, an increase in fluorescent intensity was observed for both Mb solutions added with hexenal ($P < 0.05$), whereas negligible changes were found in the controls and solutions containing hexanal ($P > 0.05$). Thereafter, fluorescent intensities of all samples gradually increased as the incubation time increased ($P < 0.05$). Hexanal and hexenal had a great impact on conformational changes of oxyMb and metMb as indicated by the higher fluorescent intensity after incubation for 2 days ($P < 0.05$). In the presence of aldehyde, unfolding of globin might be enhanced, causing the considerable exposure of tryptophan residues. A more susceptibility to structural changes was found in oxyMb, compared with metMb. Maheswarappa *et al.* (2009) reported that alteration of Mb native form in adduction with aldehyde reduced the redox stability of oxyMb. Therefore, the conformational changes of globin induced by hexanal or hexenal was more pronounced in ferrous Mb which was sensitive to oxidation.

5.4.2.3 Changes in aggregation ratio

Aggregation ratio of oxyMb and metMb solutions added with hexanal and hexenal as a function of time is shown in Figure 25B. The increase in aggregation ratio of all samples was observed with increasing incubation time up to 8 days ($P < 0.05$), except for the control metMb which had a constant value after 1 day of incubation time ($P > 0.05$). For both Mb solutions, no difference in aggregation ratio was found between Mb without and with the addition of aldehydes during 6 days of incubation ($P > 0.05$). At day 8, both Mb solutions incorporated with hexanal or hexenal had a higher aggregation ratio, compared with the control ($P < 0.05$). The result implied the profound impact of hexanal and hexenal on conformational changes of Mb with extended time. In addition, aggregation ratio of oxyMb was generally greater than metMb counterpart ($P < 0.05$). Libondi *et al.* (1994) reported that the occurrence of intermolecular cross-linking in protein was induced by malondialdehyde, accompanied by the change in the secondary structure. Therefore, aggregation of Mb was governed by the presence of aldehyde as well as the reaction time.

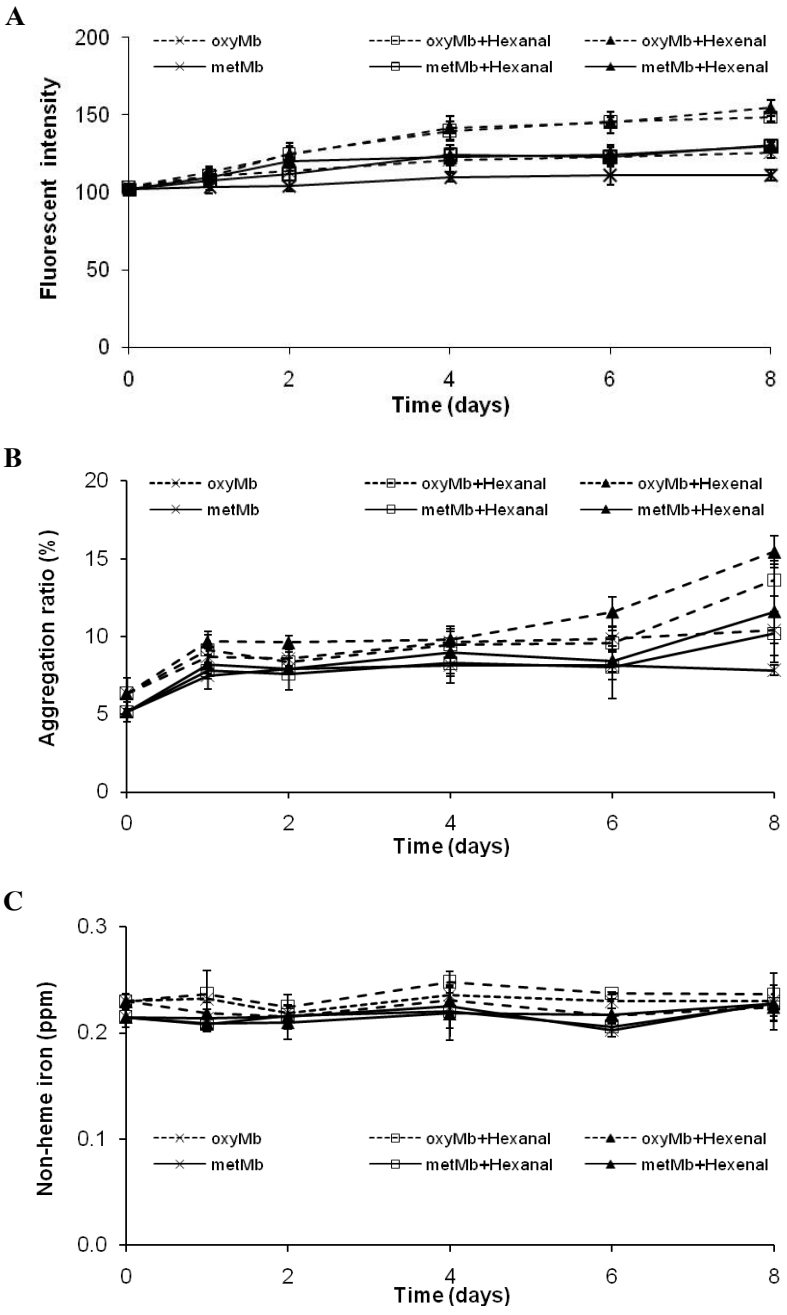


Figure 25. Effect of hexanal and hexenal on tryptophan fluorescent intensity (A), aggregation ratio (B) and non-heme iron content (C) of oxyMb and metMb solutions from Eastern little tuna dark muscle as the function of incubation time. Bars represent the standard deviation (n=3)

5.4.2.4 Changes in non-heme iron content

The effect of hexanal and hexenal on changes in non-heme iron content of oxyMb and metMb solutions at varying times is presented in Figure 25C. As the incubation time increased, no remarkable changes in non-heme iron content were obtained in all Mb, irrespective of aldehydes ($P > 0.05$). The result demonstrated that aldehydes, both hexanal and hexenal, had no effect on iron released from heme complex. Heme iron is normally cleft within porphyrin ring of heme pocket and is localized far from the surface of Mb molecule (Livingston and Brown, 1981). Although, the conformational changes of globin occurred to some extent, it had a less impact on iron release from porphyrin ring of Mb.

5.4.2.5 Changes in protein pattern

OxyMb and metMb from Eastern little tuna dark muscle containing hexanal or hexenal at a concentration of 12.5 mM were determined for protein patterns using SDS-PAGE as shown in Figure 26. The obvious band appeared at molecular weight around 15 kDa was presumed to be a Mb. Additionally, a band with molecular weight of 30 kDa was observed under the non-reducing condition in the control oxyMb and metMb (without addition of aldehydes). This protein was most likely stabilized by the disulfide bond and might be a dimer of Mb. Hexanal had no effect on the changes in protein pattern of both Mb. This might be owing to the negligible cross-linking effect of hexanal at level used in this study. Proteins with the molecular weight of 30 kDa and the higher molecular weight components were observed in oxyMb and metMb solutions added with hexenal (12.5 mM) under the reducing and non-reducing conditions. This result suggested that hexenal could induce the cross-linking of Mb via disulfide and non-disulfide covalent bonds. On the other hand, no changes in protein pattern were observed when aldehydes were incorporated at a low concentration (0.5 mM) (data not shown). Lee *et al.* (2003a) reported a mono-adduct of 4-hydroxynonenal, a reactive aldehyde, to tuna Mb by covalent modification which was expected to accelerate the oxidation of oxyMb.

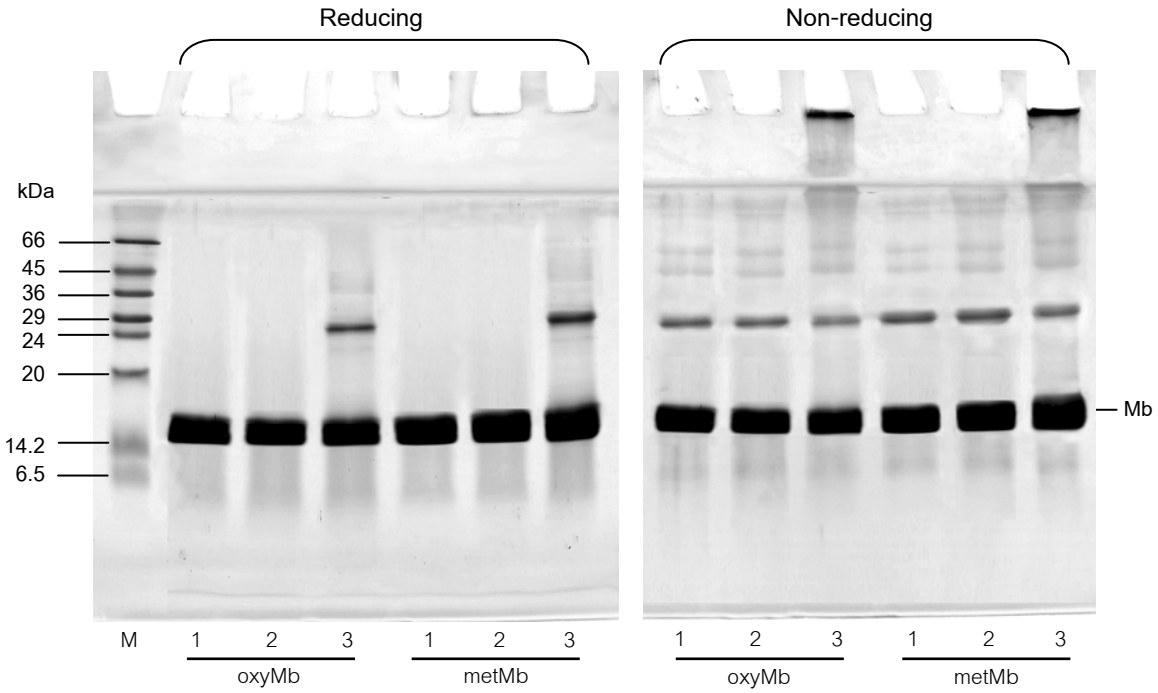


Figure 26. SDS-PAGE pattern of oxyMb and metMb solutions from Eastern little tuna dark muscle containing 12.5 mM hexanal or hexenal at 4ºC for 1 day under reducing and non-reducing conditions. M, low range markers; 1, control Mb; 2, Mb added with hexanal; 3, Mb added with hexenal

5.5 Conclusions

The oxidation of oxyMb or metMb from the dark muscle of Eastern little tuna mediated by H_2O_2 resulted in the generation of ferrylMb. $Fe^{2+}$and/or H_2O_2 could induce the conformational changes of globin without aggregation of Mb. H_2O_2 might weaken the porphyrin ring, leading to release of non-heme iron from Mb. Hexanal and hexenal caused structural changes of globin but had no effect on the release of non-heme iron. Hexenal had a greater impact on the formation of metMb, compared with hexanal, and induced the formation of covalent cross-links of Mb. Fenton's reactants and aldehyde could therefore accelerate the changes of fish Mb, however it depended on its forms.

CHAPTER 6

Effect of myoglobin from Eastern little tuna muscle on lipid oxidation of washed Asian seabass mince at different pH conditions

6.1 Abstract

The effect of pH (6.0, 6.5 and 7.0) on lipid oxidation in washed Asian seabass (*Lates calcarifer*) mince mediated by oxymyoglobin (oxyMb) from the dark muscle of little Eastern tuna (*Euthynnus affinis*) during 8 days of refrigerated storage was studied. Metmyoglobin (metMb) formation and discoloration increased with increasing storage time and the changes were more pronounced at lower pH. The highest lipid oxidation and off-odor development were observed when Mb was incorporated in washed mince at pH 6.0. At low pH, oxidation of Mb took place and lipid oxidation in washed mince was enhanced. This was concomitant with the increased fishy and rancid off-odor in the sample containing Mb, especially at pH 6.0. Washed mince containing Mb at pH 6.0 had 1-octen-3-ol and hexanal as the major volatile compounds. Thus, post-mortem pH and Mb played an essential role in lipid oxidation and off-odor in fish muscle during the extended storage.

6.2 Introduction

Lipid oxidation is associated with the development of undesirable odor and poor quality of fish and fish products, thereby affecting consumer acceptability. Lipid oxidation is a chain reaction consisting of initiation, propagation and termination reactions (Nawar, 1996). Hydroperoxide is a primary oxidation product which is readily decomposed to a variety of volatile compounds including aldehydes, ketones and alcohols (Sohn *et al.*, 2005; Varlet *et al.*, 2006). Human olfactory receptors usually have remarkably low organoleptic thresholds to most of these volatile compounds (Varlet *et al.*, 2006).

Myoglobin (Mb) is a major pigment in tuna and other dark-fleshed fish. The dark muscle of yellowfin tuna (*Neothunnus macropterus*) contains Mb ranging from 5.3 to 24.4 mg/g, while hemoglobin (Hb) ranges from 0.5 to 3.8 mg/g (Brown, 1962). However, Matsuura and Hashimoto (1954) found that Hb contents in both dark and ordinary muscle of fishes are generally greater than Mb contents. Mb has an impact on color, which is preferred by consumers. Apart from the role of oxygen storage, Mb has recently been classified as a biochemical reactor and is involved in free radical processes in aerobic organisms (Garry *et al.*, 2000). Immediately after death, Mb is mostly present in the reduced form ($MbFe^{2+}$), which provides the desirable red pigmentation to the muscle. With increasing storage time, the iron atom in the heme ring is further oxidized to the ferric state ($MbFe^{3+}$), resulting in the development of brown color (Baron and Andersen, 2002). The converting process of ferrous Mb into ferric metMb is termed 'autoxidation', which is responsible for the discoloration of meat and acceleration of lipid oxidation (Baron and Andersen, 2002; Lee *et al.*, 2003). Lee *et al.* (2003) reported that surface metMb accumulation and lipid oxidation of refrigerated tuna steaks increased during 6 days of storage. Sensory evaluation has demonstrated increased discoloration and a decrease in odor acceptability over time. pH has been known to be one of the factors governing the autoxidation of Mb (Chaijan *et al.*, 2007). Low pH was able to reduce the oxygen affinity of Hb and increased the autoxidation rate, known as the Bohr effect (Binotti *et al.*, 1971). The increasing autoxidation rate constant was found in milkfish (*Chanos chanos*) Mb with decreasing pH to be in the range of 5.5-7.0 (Chen and Chow, 2001). Susceptibility of Mb toward oxidation is affected by many factors including species. Kitahara *et al.* (1990) found that bigeye tuna oxyMb was much more prone to oxidation over the whole range of pH (pH 5-12), compared with sperm whale oxyMb.

In post-mortem fish, glycolysis leads to the accumulation of lactic acid, which in turn lowers the pH of the muscle. On the other hand, the increase in pH of fish muscle with extended storage time is due to an increase in volatile bases produced by either endogenous or microbial enzymes (Benjakul *et al.* 2002). The varied pH values in post-mortem fish can be influenced by many factors such as species, size and the handling process (Benjakul *et al.*, 2002; Masniyom *et al.*, 2002; Rawdkuen *et al.*, 2008). Masniyom *et al.* (2002) reported the pH of Asian seabass (*Lates calcarifer*) slices to range from 6.8 to 7.1 during 9 days of storage. For giant catfish (*Pangasianodon gigas*), the pH was approximately 6.3-6.6 during 14 days of refrigerated

storage (Rawdkuen *et al.*, 2008). Nevertheless, little information regarding Mb-mediated lipid oxidation as influenced by pH and its impact on the odor of fish has been reported. Tuna has been known to provide one of the most remarkable red muscle, in which Mb is found at high concentrations (Thiansilakul *et al.*, 2011). Eastern little tuna (*Euthynnus affinis*) is a species available in the Gulf of Thailand and the Indian Ocean with the volume of 45,600 metric tons and a value of 37 million US dollars in 2007 and provides the high global economic value (Fisheries Foreign Affairs Division, 2007). To study the effect of Eastern little tuna Mb on lipid oxidation in fish muscle, Asian seabass which has low content of Mb (Thiansilakul *et al.*, 2010) was used as a model system for avoiding the retained Mb. The objectives of this investigation were to assess the pro-oxidative characteristic of Mb from Eastern little tuna dark muscle in washed Asian seabass mince over a pH range of 6.0-7.0 and to study the effect of Mb on off-odor in washed mince at different pH conditions.

6.3 Materials and Methods

6.3.1 Chemicals

Triton X-100, streptomycin sulfate, 2-thiobarbituric acid and 1,1,3,3-tetramethoxypropane were purchased from Sigma (St. Louis, MO, USA). Cumene hydroperoxide was procured from Fluka (Buchs, Switzerland). Sodium dithionite, ammonium thiocyanate and ferrous chloride were obtained from Riedel (Seelze, Germany). Sodium chloride, trichloroacetic acid, chloroform and methanol were procured from Merck (Damstadt, Germany). All chemicals used were of analytical grade.

6.3.2 Fish samples

Eastern little tuna (*E. affinis*) with an average weight of 0.5-0.55 kg off-loaded after 24-36 h of capture, were obtained from the dock in Songkhla province, Thailand. Live Asian seabass (*L. calcarifer*) having an average weight of 0.5-0.55 kg from a farm in Koyo Island, Songkhla province, Thailand, were ice-shocked after capture. The fish were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla, within 1 h. Upon arrival, fish were washed with cold tap

water (5ºC), filleted and de-skinned. For Eastern little tuna, the dark muscle was manually excised and pooled. Only ordinary muscle of Asian seabass was collected. Each sample was minced with grinder (Panasonic, MK-5080M, Matsushita Electric Co., Selangor, Malaysia) until uniformity was obtained. The samples were placed on ice during preparation but not longer than 2 h.

6.3.3 Purification of Mb from Eastern little tuna dark muscle

Extraction and purification of Mb were performed according to the method of Thiansilakul *et al.* (2011). Eastern little tuna mince (100 g) was mixed with 300 ml of cold extracting medium (10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and 25 g/l Triton X-100). The mixture was homogenized for 1 min at 13,000 rpm using an IKA Labortechnik homogenizer (Selangor, Malaysia). After centrifugation at 9,600*g* for 10 min at 4ºC using an Avanti J-E centrifuge (Beckman Coulter, Palo Alto, CA, USA), the supernatant was filtered through Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK). The filtrate, referred to as "crude Mb extract", was subjected to ammonium sulfate fractionation (65-100% saturation). The precipitate obtained after centrifugation at 20,000*g* for 60 min was dissolved in a minimal volume of cold 5 mM Tris-HCl buffer, pH 8.5, which was referred to as "starting buffer". The mixture was then dialyzed using dialysis tube with molecular weight cut off of 14 kDa against 10 volumes of the same buffer with 20 changes at 4ºC. The dialysate was immediately applied onto a Sephadex G-75 column (2.6 × 70 cm; Amersham Bioscience, Uppsala, Sweden), which was previously equilibrated with the starting buffer. The separation was conducted using the starting buffer at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and measured at 280 and 540 nm to monitor the proteins and Mb in the collected fractions, respectively using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The fractions in the peak of the absorbance at 540 nm (absorbance approximately 1 to 2.5) were pooled and used as "tuna Mb".

6.3.3.1 Preparation of oxyMb

OxyMb was prepared according to the method of Tang *et al.* (2004) with some modifications. An aliquot (30 ml; 10 mg Mb/ml) of tuna Mb was added with 0.6 g of sodium dithionite with gentle stirring for 5 min to reduce all Mbs to the oxy form. The sodium dithionite was removed by dialysis of the sample against 10 volumes of cold 50 mM phosphate buffer (pH

7.0) with 10 changes of dialysis buffer at 4ºC using the dialysis tube with molecular weight cut-off of 14 kDa.

6.3.3.2 Determination of Mb concentration

The concentration of all redox forms of Mb was determined by measuring the absorbance at 525 nm which is the isobestic point for deoxyMb, oxyMb and metMb (Tang *et al.*, 2004). The molar extinction coefficient of 7.6×10^{-3} and a molecular weight of 16,110 were used in the calculation (Gomez-Basauri and Regenstein, 1992; Tang *et al.*, 2004). Mb content was expressed as mg Mb/ml.

6.3.4 Preparation of washed mince

Washed mince of Asian seabass was prepared following the method of Grunwald and Richards (2006) with slight modification. The mince was washed twice with cold distilled water with a mince/water ratio of 1:3 (w/v). The mixture was stirred continuously for 2 min with a glass rod and allowed to settle for 15 min. The mixture was filtered using 2 layers of cheese-cloth. Thereafter, 3 volumes of cold 50 mM sodium phosphate buffer with different pH (6.0, 6.5 and 7.0) were mixed with washed mince, stirred and filtered as described previously. The retentate was mixed with a designated buffer with a mince/buffer ratio of 1:1 (w/v) and homogenized for 3 min at a speed of 13,000 rpm. The pH of the mixture was checked (Docu-pH meter, Satorious, USA) and adjusted to the desirable pH (pH 6.0, 6.5 and 7.0) using 0.5 M NaOH or 0.5 M HCl. After standing for 15 min, the mixture was centrifuged at 15,000*g* for 25 min at 4ºC. The pellet was collected and referred to as "washed mince". The moisture contents of all washed mince samples were determined and adjusted to 85% using the corresponding buffer.

6.3.5 Effect of Mb on lipid oxidation in washed mince at different pH

To study the impact of Mb on lipid oxidation, oxyMb from Eastern little tuna was added into washed mince having a different pH (6.0, 6.5 or 7.0) to obtain a final concentration of 0.2 g Mb/100g sample. For the control samples, the same volume of distilled water was added instead of oxyMb. In order to inhibit the microbial growth, streptomycin sulfate was added to the washed mince to obtain a final concentration of 200 ppm and mixed thoroughly. The samples were individually packed in polyethylene zip-lock bags with the dimension of 3"×4"

(Ziploc®, S. C. Johnson & Son Ltd., Bangkok, Thailand) without headspace and stored at 4ºC for 8 days. During storage, 3 bags of each treatment were randomly taken at day 0, 2, 4, 6 and 8 for analyzes.

6.3.5.1 Measurement of Mb oxidation

Mb in washed mince samples was extracted following the method of Benjakul and Bauer (2001). Washed mince (2 g) was weighed and transferred into a 50-ml polypropylene centrifuge tube and 20 ml of cold 40 mM phosphate buffer (pH 6.8) was added. The mixture was homogenized at 13,500 rpm for 10 s, followed by centrifuging at 3,000*g* for 30 min at 4ºC. The supernatant was filtered through Whatman No. 1 filter paper. Mb solutions were measured for absorption spectra ranging from 350 to 750 nm to monitor Mb oxidation by which the wavelength maxima at 503, 557 and 582 nm represented for metMb, deoxyMb and oxyMb, respectively. The proportion of metMb was calculated following a modified Krzywicki's equation (Tang *et al.*, 2004) as followed:

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

where $R_1 = A_{582}/A_{525}$, $R_2 = A_{557}/A_{525}$ and $R_3 = A_{503}/A_{525}$.

6.3.5.2 Measurement of color

Colorimetric values of washed mince were determined using a colorimeter (JP7100F, Juki Corp., Tokyo, Japan). The instrument was calibrated using a white and black standard. L^* (lightness), a^* (redness/greenness) and b^* (yellowness/blueness) were determined.

6.3.5.3 Measurement of peroxide value (PV)

The peroxide value (PV) which generally increases in the early stage of lipid oxidation was determined as described by Richards and Hultin (2000) with some modifications. Washed mince (1 g) was mixed with 11 ml of a chloroform/methanol mixture (2:1, v/v). The mixture was homogenized at 13,500 rpm for 2 min and then filtered using Whatman No. 1 filter paper. To the filtrate (7 ml) was added 2 ml of 0.5% NaCl. The mixture was vortexed at a moderate speed for 30 s using a Vortex-Genie2 mixer 4 (Scientific Industries, Bohemia, NY, USA). After centrifugation at 3,000*g* for 3 min, the sample was separated into 2 phases. To the lower phase (3 ml) were added 2 ml of cold chloroform/methanol (2:1) mixture, 25 µl of 30% (w/v) ammonium thiocyanate and 25 µl of 20 mM ferrous chloride in 3.5% HCl. The reaction mixture was allowed to stand for 20 min at room temperature and the absorbance at 500 nm was

read. A standard curve was prepared using cumene hydroperoxide at concentrations ranging from 0 to 5 ppm. PV was calculated and expressed as mg cumene hydroperoxide/kg sample.

6.3.5.4 Measurement of thiobarbituric acid-reactive substances (TBARS)

Thiobarbituric acid-reactive substances (TBARS), the secondary oxidation products, were determined following the method of Buege and Aust (1978). The ample (0.5 g) was homogenized with 2.5 ml of a solution containing 0.375% thiobarbituric acid (w/v), 15% trichloroacetic acid (w/v) and 0.25 M HCl. The mixture was heated in a boiling water bath for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3,600*g* at 25ºC 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 6 ppm. TBARS values were calculated and expressed as mg malonaldehyde/kg sample.

6.3.5.5 Measurement of volatile compounds

Volatile compounds in washed mince (pH 6.0) without and with the addition of Mb from Eastern little tuna, stored at 4ºC for 8 days, were determined using solid-phase micro-extraction gas chromatography mass spectrometry (SPME-GCMS) (Iglesias and Medina, 2008). SPME is solvent free technique, makes the fast separation and can yield the low range in detection limit (Pawliszyn *et al.*, 1997).

Extraction of volatile compounds by SPME fiber

To extract volatile compounds, 3 g of sample was mixed with 8 ml of deionized water and homogenized at a speed of 13,500 rpm for 2 min. The mixture was centrifuged at 2,000*g* for 10 min. The supernatant (6 ml) was heated at 60ºC in a 20-ml headspace vial with equilibrium time of 10 h. The SPME fiber (50/30 µm DVB/Carboxen™/PDMS StableFlex™) (Supelco, Bellefonte, PA, USA) was conditioned at 270ºC for 15 min before use and then exposed to the headspace. The 20 ml-vial (Agilent Technologies, Palo Alto, CA, USA) containing the sample extract and the volatile compounds were allowed to adsorb into the SPME fiber at 60ºC for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270ºC.

GC-MS analysis

GC-MS analysis was performed in a HP 5890 series II gas chromatograph coupled with HP 5972 mass-selective detectors equipped with a splitless injector and coupled

with a quadrupole mass detector (Hewlett Packard, Atlanta, GA, USA). Compounds were separated on a HP-Innowax capillary column (Hewlett Packard) (30 m × 0.25 mm ID, with film thickness of 0.25 μm). The GC oven temperature program was: 35ºC for 3 min, followed by an increase of 3ºC/min to 70ºC, then an increase of 10ºC/min to 200ºC and finally an increase of 15ºC/min to a final temperature of 250ºC and holding for 10 min. Helium was employed the carrier gas, with a constant flow of 1 ml/min. The injector was operated in the splitless mode and its temperature was set at 270ºC. Transfer line temperature was maintained at 265ºC. The quadrupole mass spectrometer was operated in the electron ionization (EI) mode and source temperature was set at 250ºC. Initially, full-scan-mode data were acquired to determine appropriate masses for the later acquisition in scan mode under the following conditions: mass range: 25-500 μ and scan rate: 0.220 s/scan. All the analyzes were performed with ionization energy of 70 eV, filament emission current at 150 μA and the electron multiplier voltage at 500 V.

Analyzes of the volatile compounds

Identification of the volatile compounds in the samples was based on the retention times of individual aldehydic standards including pentanal, hexanal, heptanal, octanal and nonanal. Identification of the compounds was also done by consulting ChemStation Library Search (Wiley 275.L). Quantitative determination was carried out using an internal calibration curve that was built using stock solutions of the compounds in ultra-pure water saturated in salt and analyzing them by the optimized HS-SPME method. Quantification limits were calculated to a signal-to-noise (S/N) ratio of 10. Repeatability was evaluated by analyzing 3 replicates of each sample. The identified volatile compounds related to lipid oxidation, including aldehydes, alcohols, ketones, etc., were each presented as normalized area in percent under peak of each identified compound.

6.3.5.6 Sensory evaluation

Sensory evaluation was performed by 6 trained panelists who had extensive experience in the evaluation of off-odors of raw fish. Panelists were trained with standards for two sessions using a 5-point scale ranging from none (score=0) to strong (score=4) represented for fishy and rancid odors (Sohn *et al.*, 2005). The standards were prepared by storing seabass slices (1 cm thickness) packed in the polyethylene bags in ice for 0 to 15 days representing the

score of 0 to 4. To test the samples, panelists were asked to open the sealable polyethylene bags containing the test samples and sniff the headspace above the samples.

6.3.6 Statistical analysis

Experiments were run in triplicate using 3 different lots of samples. Data were subjected to analysis of variance (ANOVA) with the confidence level of 95%. Comparison of means was carried out by Duncan's multiple range test. For pair comparison, the Student's T-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

6.4 Results and Discussion

6.4.1 MetMb formation

OxyMb prepared from the dark muscle of Eastern little tuna (8.08% deoxyMb, 63.05% oxyMb and 29.38% metMb) was added to washed Asian seabass mince to obtain a concentration of 2 mg/g, which was the level of heme protein found in ordinary muscle of Eastern little tuna (data not shown). To ensure that most of the heme proteins including Mb were removed, a washing process was applied (Grunwald and Richards, 2006). After washing, the lipid content in seabass mince was less than 0.41% (wet weight basis) and only negligible Mb content was found. With the addition of Mb, the washed Asian seabass mince contained approximately 2 mg Mb/g. MetMb formation in washed mince at various pH values during refrigerated storage is shown in Table 8. After 2 days of storage, the proportion of metMb increased from 29.4% to 61.7%, 59.3% and 56.6% for washed mince with pH of 6.0, 6.5 and 7.0, respectively ($P < 0.05$). The continuous increases in metMb formation were observed in all samples with increasing storage time up to day 6 ($P < 0.05$). Thereafter, metMb remained constant ($P > 0.05$). The result suggested that the higher autoxidation of Mb was more pronounced at the initial storage time. Many factors are known to contribute to autoxidation of Mb, including pH, temperature, oxygen and lipid oxidation products (Chaijan *et al.*, 2007; Faustman *et al.*, 1999; Monahan *et al.*, 2005; Thiansilakul *et al.*, 2011). In fresh meat, the reducing substances such as NAD^+, FAD^+, or endogenous enzymes are responsible for the reduction of metMb (Benjakul and Bauer, 2001;

Eder, 1996). Thus, Mb added to washed mince, in which a small amount of reducing substances was retained, might be susceptible to oxidation, as evidenced by the increased metMb formation. During iced storage of sardine (*Sardinella gibbosa*) and mackerel (*Rastrelliger kanagurta*), metMb formation tended to increase with the simultaneous decreases in redness index (Chaijan *et al.*, 2005).

At day 2 of storage, the highest metMb formation was found in washed mince with pH 6.0 in comparison with those having pH 6.5 and 7.0 ($P < 0.05$). The result suggested that autoxidation of Mb was enhanced at lower pH. According to the Bohr's effect, the proton-catalyzed displacement process was mainly responsible for promoting the autoxidation of Hb (Binotti *et al.*, 1971). This probably governed the autoxidation of Mb at acidic pH, while the oxygenation of Mb might occur at alkaline pH (Chaijan *et al.*, 2007; Thiansilakul *et al.*, 2011). Chaijan *et al.* (2007) reported the greatest metMb formation at pH 5 and 6, while decreased metMb formation was obtained at pH > 6. At day 4, the higher metMb formation was observed in washed mince with pH 6.0 and 6.5, compared with the sample with the higher pH (pH 7.0) ($P < 0.05$). However, no differences in metMb formation among all samples were noticeable after 6 days of storage until the end of storage ($P > 0.05$). Therefore, pH had an influence on metMb formation at the initial time of storage, but had no impact on metMb formation when excessive oxidation had occurred.

Table 8. The formation of metMb from Eastern little tuna in washed mince at different pH during 8 days of refrigerated storage

Storage time	metMb formation (%)		
(days)	pH 6.0	pH 6.5	pH 7.0
0	$29.38 \pm 0.77^{dA*}$	29.38 ± 0.77^{dA}	29.38 ± 0.77^{dA}
2	61.70 ± 0.37^{cA}	59.24 ± 0.88^{cB}	56.54 ± 1.07^{cC}
4	79.21 ± 0.74^{bA}	77.65 ± 0.17^{bA}	72.20 ± 1.41^{bB}
6	87.91 ± 0.46^{aA}	86.80 ± 0.71^{aA}	87.52 ± 0.54^{aA}
8	88.00 ± 0.68^{aA}	86.97 ± 0.78^{aA}	87.65 ± 0.04^{aA}

* Different superscripts in the same column indicate significant differences ($P < 0.05$). Different capital superscripts within the same row indicate significant differences ($P < 0.05$).

6.4.2 Changes in redness

Changes in redness (*a**-value) of washed mince without and with the addition of Mb during refrigerated storage are shown in Figure 27. Differences in redness were observed in washed mince with varying pH ($P < 0.05$). At day 0, washed mince containing Mb with pH 7.0 exhibited the highest redness, followed by those having pH 6.5 and 6.0, respectively ($P < 0.05$). Roth *et al.* (2009) revealed a correlation between pH and color of Atlantic halibut (*Hippoglossus hippoglossus*) flesh, in which decreased pH caused a decrease in *a**-value. Additionally, the decrease in redness was coincidental with increased metMb content (Lee *et al.*, 2003; Rawdkuen *et al.*, 2008; Sohn *et al.*, 2005). Our results indicated that the low pH might induce Mb oxidation to a greater extent, leading to a discoloration of washed mince as shown by the lowered *a**-value. After 2 days of storage, the decreases in redness of all samples containing Mb were noticeable ($P < 0.05$). The continuous decreases in redness of washed mince with pH 6.5 and 7.0 were found up to 6 days of storage ($P < 0.05$), whereas no change in redness was observed in the pH 6.0 sample after 4 days of storage ($P > 0.05$). At the initial time of storage, when Mb oxidation proceeded to a higher rate as evidenced by higher metMb formation (Table 8), a marked decrease in redness of washed mince was noticeable. With extended storage time, a lower rate of Mb oxidation occurred (Table 8), resulting in a negligible change in redness. The control washed mince with pH 6.0, 6.5 and 7.0 (without Mb addition) had low *a**-values (-1.01 to -0.70) throughout the refrigerated storage of 8 days. Heme proteins contributing to color of muscle could be removed during the washing process, resulting in nondetectable redness of washed mince.

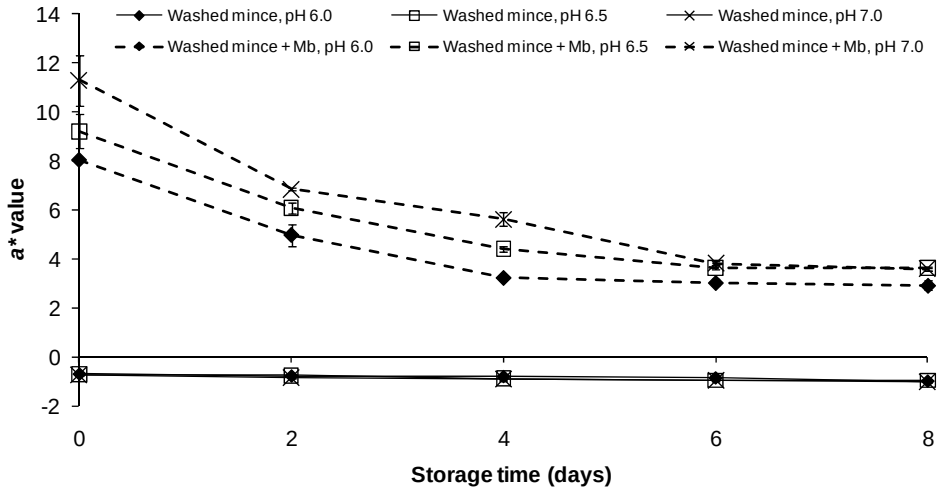


Figure 27. Changes in *a**-value of washed mince without and with the addition of Mb (2 mg/g) from Eastern little tuna at different pH values during 8 days of refrigerated storage. Bars represent the standard deviation (n=3)

6.4.3 Changes in PV

Changes in PV of washed mince without and with the addition of Mb at various pH conditions were monitored during 8 days of refrigerated storage as depicted in Figure 28A. For the control washed mince (without Mb), no changes in PV were found throughout the storage of 8 days ($P > 0.05$), except for the control with pH 6.0, which had a slight increase in PV at day 8 ($P < 0.05$). Generally, lipid oxidation of depot and membrane lipids occurs in fish muscle during extended storage (Thiansilakul *et al.*, 2010). Nevertheless, washing could remove some pro-oxidants as well as some lipids from the muscle (Grunwald and Richards, 2006). As a result, negligible lipid oxidation occurred in washed mince. The slight increase in PV of washed mince at pH 6.0 might be caused by the accelerated oxidation of membrane phospholipids at pH 6.0. In the presence of Mb, washed mince with pH 6.0, 6.5 and 7.0 exhibited marked increases in PV up to 4 days of storage and decreases in PV were obtained thereafter ($P < 0.05$). The results revealed a pronounced impact of Mb added into washed mince on the enhancement of lipid oxidation. During lipid oxidation, hydroperoxides are generated (Sohn *et al.*, 2005; Thiansilakul *et al.*, 2010). Decreases in PV after 4 days of storage suggested that the hydroperoxides formed might

be decomposed or transformed to other compounds (Grunwald and Richards, 2006; Nawar, 1996; Thiansilakul *et al.*, 2010).

During refrigerated storage, the highest formation of PV was found in washed mince containing Mb with pH 6.0, followed by those having pH 6.5 and 7.0, respectively ($P < 0.05$). Nevertheless, at day 8, PV of washed mince with pH 6.5 and 7.0 were similar ($P > 0.05$). Thus, lipid oxidation in washed mince with added Mb was mainly governed by pH. The formation of hydroperoxides was in accordance with the increased metMb formation (Table 8). Mb oxidation has been proposed to be related to lipid oxidation in fish muscle (Rawdkuen *et al.*, 2008; Sohn *et al.*, 2005). Autoxidation of oxymyglobin results in the formation of metmyogobin and superoxide which rapidly dismutates to H_2O_2 and oxygen (Baron and Andersen, 2002). Moreover, the interaction of H_2O_2 with metMb leads very rapidly to generation of an active species, ferrylMb, which could catalyze lipid peroxidation (Chan *et al.*, 1997b). Apart from Mb autoxidation, a low affinity of heme to globin within metMb might be related to the enhanced lipid oxidation. It was found that the higher the heme affinity of Mb, the lower the Mb-mediated lipid oxidation obtained (Richards *et al.*, 2009). Therefore, low pH was not only associated with Mb oxidation, but also weakened the heme-globin complex, leading to a release of heme group, which was able to induce the lipid oxidation.

6.4.4 Changes in TBARS

Changes in TBARS of washed mince without and with the addition of Mb at various pH values during refrigerated storage are shown in Figure 28B. During 8 days of storage, no changes in TBARS values were observed in the control washed mince, regardless of pH ($P > 0.05$). For washed mince containing Mb at various pH, the continuous increases in TBARS were found up to 6 days of storage, followed by a decrease at day 8 ($P < 0.05$). TBARS values have been used to measure the concentration of relatively polar secondary reaction products, especially aldehydes (Nawar, 1996). At day 6, a sharp decrease in PV was found, while the TBARS value increased. This suggested that the decomposition of hydroperoxides into secondary oxidation products took place at a much higher rate in comparison with the formation of hydroperoxides. The decrease in TBARS at the end of storage might be caused by a loss of low-molecular-weight

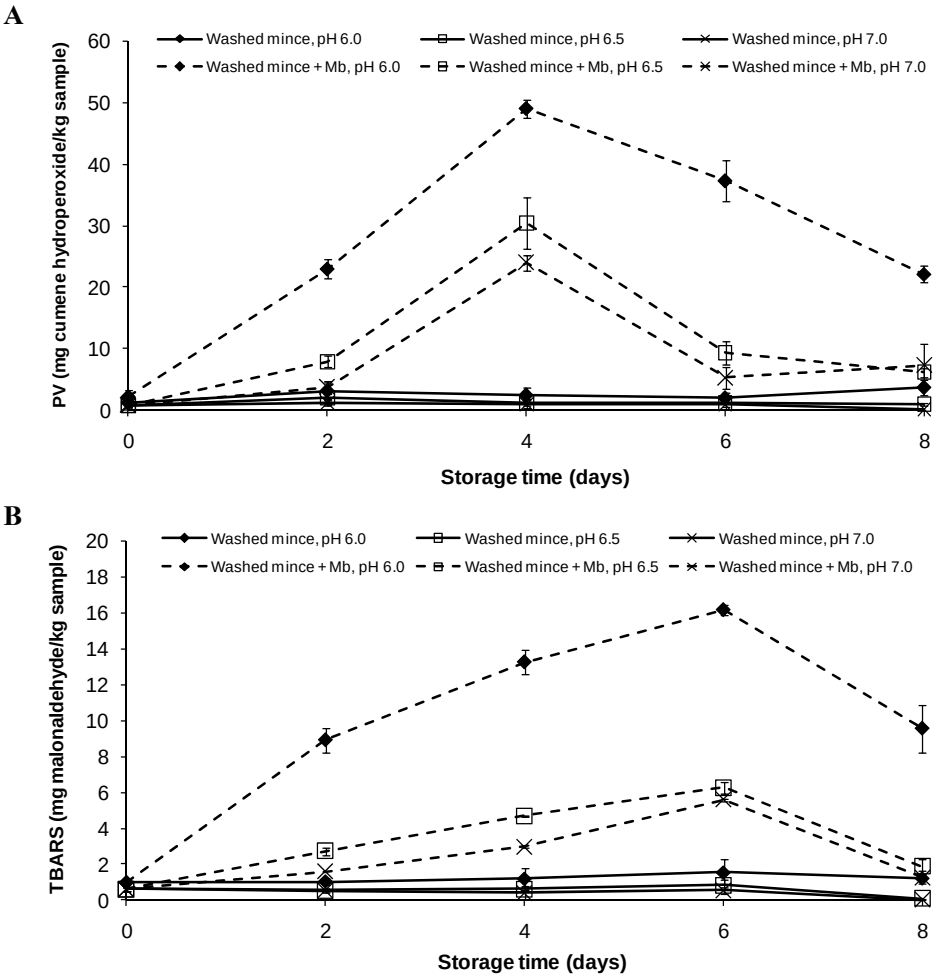


Figure 28. Changes in PV (A) and TBARS (B) of washed mince without and with the addition of Mb (2 mg/g) from Eastern little tuna at different pH values during 8 days of refrigerated storage. Bars represent the standard deviation (n=3)

products during the advancement of oxidation (Nawar, 1996). The ability of wild-type sperm whale Mb to promote the formation of lipid peroxide and TBARS was reported in washed cod muscle (Grunwald and Richards, 2006).

During storage, washed mince containing Mb with pH 6.0 generally exhibited the highest TBARS values, compared with those having pH 6.5 and 7.0 ($P < 0.05$). Thus, lipid oxidation catalyzed by Mb could be accelerated by a lower pH. It was noted that oxidation of Mb in washed mince at all pH levels was associated with lipid oxidation during the early stage of

storage. This was demonstrated by similar metMb formation among washed mince with various pH values after 6 days of storage (Table 8). Low pH might induce the rate of Mb oxidation as well as a conformational change of Mb, resulting in the promotion of lipid oxidation in washed mince. Generally, the native conformation of Mb is characterized by a heme group bound to the tightly folded protein in a hydrophobic pocket (Pegg and Shahidi, 1997). The changes in the conformation of Mb induced by hydrogen ions or hydroxyl ions could lead a susceptible heme group to autoxidation or destruction (Chaijan *et al.*, 2007; Thiansilakul *et al.*, 2011). Therefore, Mb oxidation and conformational change should be suppressed to decrease pro-oxidative activity of Mb.

6.4.5 Sensory property

Washed mince without and with the addition of Mb at varying pH was evaluated for fishy and rancid odors during 8 days of refrigerated storage as shown in Figure 29. Fishy and rancid odors have been identified as the common off-flavors associated with fish flesh and are directly related to the formation of secondary lipid oxidation products (Sohn *et al.*, 2005). At day 0, negligible fishy and rancid odors were found in washed minces irrespective of pH and Mb incorporation ($P > 0.05$). Fishy and rancid odors were more intense in all samples as storage time progressed. For the control washed mince, the intensities of fishy and rancid odors increased gradually during 8 days of storage ($P < 0.05$). At all storage times, no differences in the intensities of both odors for the control samples with different pH were observed ($P > 0.05$). This suggested that lipid oxidation still took place in washed mince without added Mb. This might be caused by the oxidation of membrane phospholipids retained in mince. Phospholipids have been reported to be susceptible to oxidation owing to the high content of PUFAs (Chan *et al.*, 1997a). Nevertheless, the intensities were generally lower than that of washed mince containing Mb throughout the storage period ($P < 0.05$). The formation of secondary lipid oxidation products is one of the main causes of the development of undesirable odors in fish muscle (Thiansilakul *et al.*, 2010). The greater lipid oxidation in samples with added Mb was coincidental with the higher intensity of both fishy and rancid odors. Sohn *et al.* (2005) reported that the changes in total lipid hydroperoxide content and TBARS of yellowtail (*Seriola quinqueradiata*) dark muscle were

accompanied with the increasing intensity of fishy odor, rancid off-odor, as well as increasing metMb formation.

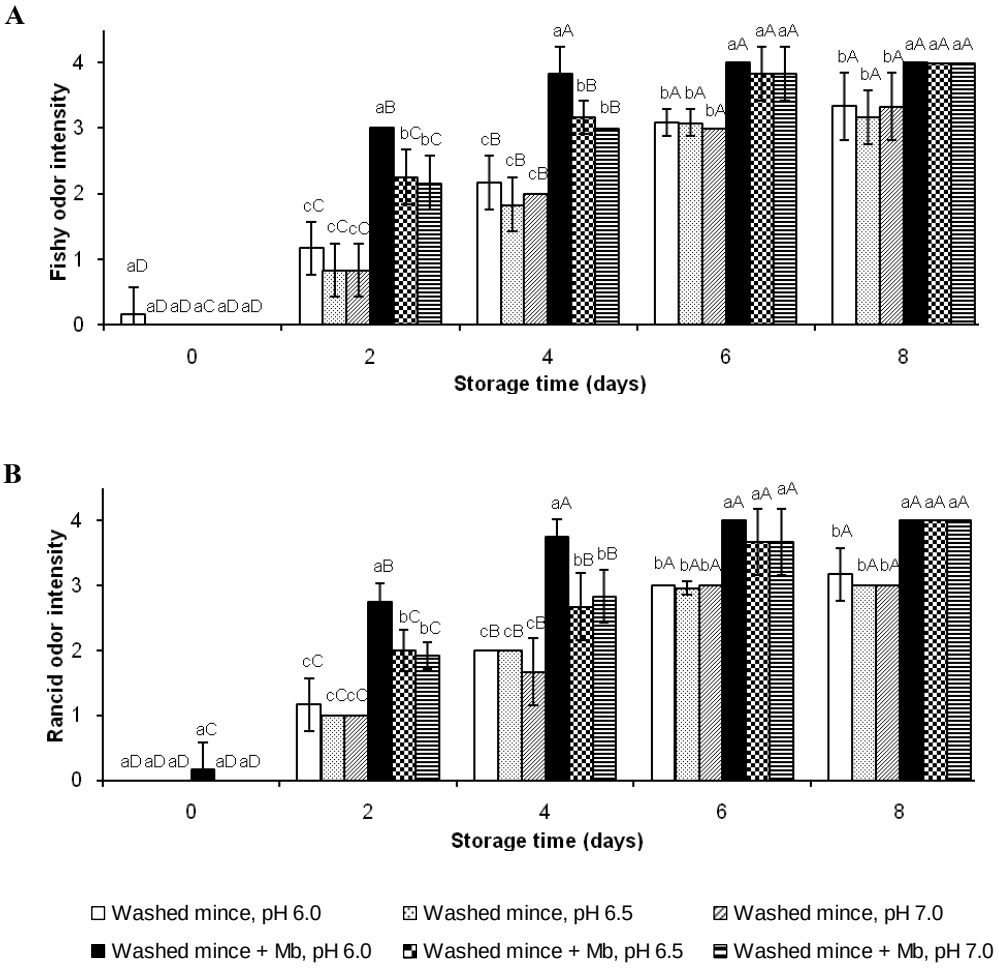


Figure 29. Changes in fishy (A) and rancid (B) odors of washed mince without and with the addition of Mb (2 mg/g) from Eastern little tuna at different pH values during 8 days of refrigerated storage. Scores range from none (score=0) to strong (score=4). Different letters on the bars within the same storage time indicate significant differences ($P < 0.05$). Different capital letters on the bars within the same sample indicate significant differences ($P < 0.05$). Bars represent the standard deviation (n=3)

In the presence of Mb, washed mince with pH 6.0 exhibited stronger fishy and rancid odors within the first 4 days of storage and had higher intensities than those of pH 6.5 and 7.0 ($P < 0.05$). However, no differences in both fishy and rancid odors among samples containing Mb with different pHs were noticeable during 6-8 days of storage ($P > 0.05$). The development of fishy and rancid odors was related to the increased TBARS, especially during the first 4 days of storage (Figure 28B). Trout hemolysate increased rancidity as well as TBARS of washed cod muscle more rapidly at pH 6.0, compared with pH 7.2. MetHb formation due to autoxidation of the heme pigment was found to take place more rapidly at reduced pH (Richards and Hultin, 2000). Therefore, the oxidation of Mb preferably occurring at low pH could play a crucial role in the generation of oxidation products associated with undesirable fishy and rancid odors of fish muscle.

6.4.6 Development of volatile compounds

Development of volatile compounds in washed mince mediated by Mb at pH 6.0 after refrigerated storage for 8 days is displayed in Table 9. In the absence of Mb, washed mince (pH 6.0) stored for 8 days contained secondary oxidation products including hexanal, 2-pentyl furan, tetradecanal and octadecanal. Both saturated and unsaturated fatty acids were found in Asian seabass muscle, in which palmitic acid (C16:0) was the most abundant fatty acid, followed by docosahexaenoic acid (C22:6(n-3)) (Thiansilakul *et al.*, 2010). Several derivatives of aldehyde, ketone and alcohol can be formed by the oxidation of unsaturated fatty acids in fish flesh (Varlet *et al.*, 2006).

When Mb was incorporated in washed mince, hexanal, 1-octen-3-ol and 2-pentyl furan were found as the dominant volatile compounds. This indicates that lipid oxidation took place to a higher extent in the sample containing Mb. Apart from hexanal, the aldehydic substances including heptanal, octanal, nonanal, E-2-octenal, benzaldehyde, ethyl benzaldehyde and pentadecanal were also detectable. Additionally, alcohol (1-octen-3-ol and 1-heptanol), ketone (2-undecanone) and the other volatile substances (octyl formate) were also generated during storage of 8 days. This suggested that Mb was able to catalyze lipid oxidation in washed mince intensively. Hexanal and 1-octen-3-ol, the major components, possibly contributed to the strong intensities of fishy and rancid off-odors in washed mince containing Mb. Odor detection

thresholds have been reported to be 10.5 ppb for hexanal and 1 ppb for 1-octen-3-ol (Buttery *et al.*, 1988; Milo and Grosch, 1993). Varlet *et al.* (2006) reported that carbonyl compounds involving 4-heptenal, octanal, decanal and 2,4-decadienal were responsible for fishy odor in salmon flesh (*Salmo salar*). The fishy volatiles identified in boiled sardines were dimethyl sulfide, acetaldehyde, propionaldehyde, butyraldehyde, 2-ethylfuran, valeraldehyde, 2,3-pentanedione, hexanal and 1-penten-3-ol (Kasahara and Osawa, 1998). Therefore, the development of volatile compounds coincidentally occurred with the enhanced lipid oxidation. The oxidation products of phosphatidylcholine liposomes and the addition of known oxidation products of oleic and linoleic acids (2-octene, propanal, decanal, nonanal, hexanal, 2-nonenal and 2-heptenal) could accelerate oxyMb oxidation (Chan *et al.*, 1997a). It was noted that some volatile compounds might be lost after 8 days of storage. Therefore, the volatile compounds detected more likely represented those retained in the intact muscle. Therefore, to obtain fish with retarded formation of off-odor during extended storage, oxidation of Mb should be suppressed.

Table 9. Volatile compounds in washed mince without and with the addition of Mb (2 mg/g) from Eastern little tuna at pH 6.0 after 8 days of refrigerated storage

Compounds	Concentration (%)	
	Washed mince	Washed mince + Mb
Hexanal	2.52	11.00
Heptanal	ND	2.02
2-pentyl furan	1.29	7.08
Octanal	ND	2.61
Nonanal	ND	3.93
E-2-Octenal	ND	1.91
1-Octen-3-ol	ND	11.54
1-Heptanol	ND	1.31
Benzaldehyde	ND	1.43
Octyl formate	ND	1.60
2-Undecanone	ND	3.09
Ethyl benzaldehyde	ND	0.73
Tetradecanal	0.35	ND
Pentadecanal	ND	1.12
Octadecanal	0.81	ND

ND : non-detectable

6.5 Conclusion

During refrigerated storage, washed mince containing Mb at various pH conditions underwent discoloration, lipid oxidation, as well as off-odor development to a greater extent, compared with the controls (without Mb). These changes were more likely associated with metMb formation occurring in washed mince as storage time increased. pH had a profound impact on the pro-oxidative activity of Mb in which greater lipid oxidation occurred at the lower pH. Mb-mediated lipid oxidation could develop intense fishy and rancid off-odors in washed mince, especially at low pH. Lipid oxidation products including aldehyde, ketone and alcohol

most likely contributed to off-odors development in washed mince catalyzed by Mb. In order to maintain the quality of fish, especially those with high content of Mb, lowering of pH should be avoided by shortening the handling or storage time. Retardation of glycolysis or anaerobic respiration related with pH lowering should be a means to prevent such a problem in fish flesh.

CHAPTER 7

Characteristics of myoglobin and hemoglobin-mediated lipid oxidation in washed mince from bighead carp (*Hypophthalmichthys nobilis*)

7.1 Abstract

Myoglobin (Mb) and hemoglobin (Hb) accounted for 61% and 39% of the total heme protein extracted from bighead carp (*Hypophthalmichthys nobilis*) dark muscle, respectively. Molecular weight of Mb was 16,445 Da whereas Hb comprised two α-chains (16,006 Da) and two β-chains (16,104 and 16,180 Da). Hemin loss from metHb was rapid at pH 6.0 (4ºC). For metMb, hemin loss increased as pH decreased to 5.5. Pro-oxidative activities of oxyMb/Hb and metMb/Hb were examined in washed mince at pH 6.0 during 9 days of iced storage. Soret measurement suggested that oxyMb and metMb remained intact as holoMb throughout storage and some cross-linked Mb occurred from metMb. For oxyHb and metHb, weakening of the heme-globin linkage was observed, especially for metHb which had undetectable Soret after 3 days of storage. Loss of redness in washed mince containing Hb was more rapid and extensive, compared to that of Mb. During storage, Hb promoted the greater peroxides, thiobarbituric acid-reactive substances and hexanal than did Mb ($P < 0.05$) and met-form was likely a stronger pro-oxidant than oxy-form. Thus, Hb which appeared rooted in relatively low hemin affinity and high autoxidation was superior in promoting lipid oxidation compared to Mb.

7.2 Introduction

Lipid oxidation in meat is accentuated during handling, processing, storage and cooking. This undesirable reaction lowers the quality and acceptability of meats and other muscle products. Various pro-oxidants have the potential to promote lipid oxidation in muscle food systems (Nawar, 1996). The heme proteins, both myoglobin (Mb) and hemoglobin (Hb), have

been shown to promote lipid oxidation in model systems, especially at low pH (Grunwald and Richards, 2006; Thiansilakul *et al.*, 2011a).

Mb and Hb are monomeric and tetrameric heme proteins, respectively. They contain iron in a prosthetic heme group. Hb, which transports oxygen and other gases, is the main protein in blood and highly concentrated in the erythrocytes (Jensen, 2004). Blood capillaries can burst naturally and also during mechanical treatments such as filleting and mincing, leading to the distribution of Hb throughout the muscle. Mb, the oxygen-storage protein retained by the intracellular structure, is a major pigment in the dark muscle of fish (Chaijan *et al.*, 2004; Thiansilakul *et al.*, 2011b). Heme proteins contribute to the color of muscle, depending upon its derivatives and concentration (Faustman and Cassens, 1990). The content of Mb and Hb in fish depends on muscle types and fish species (Brown, 1962; Chaijan *et al.*, 2004).

Immediately after death, Mb and Hb are mostly present in the reduced form or the ferrous state (Mb/Hb-Fe^{2+}), which provides the desirable red pigmentation to the muscle. With increasing storage time, the iron atom in the heme ring is further oxidized to the ferric state (Mb/Hb-Fe^{3+}), resulting in the development of brown color (Faustman and Cassens, 1990). H_2O_2 occurred during met-heme protein formation can further generate ferryl-heme protein (Mb/Hb-Fe^{4+}), a strong pro-oxidant (Kanner and Harel, 1985). Autoxidation of heme protein is responsible for the discoloration of meat and acceleration of lipid oxidation (Faustman and Cassens, 1990; Thiansilakul *et al.*, 2011a). Lipid oxidation in washed Asian seabass mince initiated by Mb brought about the increased fishy and rancid off-odor, especially at low pH, in which metMb was the dominant form (Thiansilakul *et al.*, 2011a). Pro-oxidative activity of Hb from different fish species, including Asian seabass, tilapia and grouper, varied depending on the molecular properties of Hb (Maqsood and Benjakul, 2011). Richards *et al.* (2005) reported autoxidation and hemin loss of trout Mb and Hb associated with lipid oxidation in washed fish muscle at pH 6.3. Hemin loss rate was considered more crucial in promoting lipid oxidation, compared to autoxidation rate. Trout Hb was prone to hemin loss and became a stronger promoter of lipid oxidation, while trout Mb had a more rapid autoxidation rate (Richards *et al.*, 2005). Released hemin readily converts pre-formed lipid hydroperoxides to radicals that facilitate lipid oxidation (Van Der Zee *et al.*, 1996).

Dark fleshed fish, containing substantial quantities of Mb and Hb in the dark muscle, are considered to be highly prone to discoloration and lipid oxidation. However, little has been reported on the relative efficacy of Mb and Hb to promote lipid oxidation. A better understanding of lipid oxidation initiated by these heme proteins would provide basic knowledge, which leads to preventive methods to inhibit lipid oxidation and improve overall quality of fish. The objective of this investigation was to comparative study the pro-oxidative activity of bighead carp Mb and Hb, both oxy- and met-forms, in washed bighead carp mince during iced storage.

7.3 Materials and Methods

7.3.1 Chemicals

Triton X-100, sodium dodecyl sulphate (SDS), β-mercaptoethanol (βME), sodium dithionite and ferrous chloride were procured from Fisher Scientific (Pittsburgh, PA, USA). Streptomycin sulphate, ammonium thiocyanate, 2-thiobarbituric acid, cumene hydroperoxide and 1,1,3,3-tetramethoxypropane were purchased from Sigma (St. Louis, MO, USA). All chemicals were of analytical grade.

7.3.2 Collection of fish samples and blood

Blood from 55 juvenile bighead carp (*H. nobilis*) (7-8 cm in length) were obtained from the Columbia Environmental Research Center (Columbia, MO, USA). Blood and fillets from adult bighead carp (50-60 cm in length) were obtained from Schafer Fisheries (Thomson, IL, USA). Blood was removed from the caudal vein of newly killed carp by severing the caudal peduncal and collecting blood in sodium heparin (30 Units heparin per ml of blood drawn). The fish fillets and heparinized blood were placed in an insulated box containing frozen gel packs and transported to the Meat Science and Muscle Biology Laboratory, University of Wisconsin-Madison (Madison, WI, USA) within 1 day after capture. Upon arrival, dark and ordinary muscles were separated and cut into small pieces. Samples were ground using a KSM90WW household mixer (Kitchen Aid, Inc., St. Joseph, MI, USA) until uniformity was obtained. Mince from dark and ordinary muscle was used for preparation of Mb and washed mince, respectively, whereas heparinized blood was used for Hb preparation.

7.3.3 Preparation of heme proteins with oxy- and met-forms

7.3.3.1 Preparation of Mb

Extraction and purification of Mb were performed according to the method of Thiansilakul *et al.* (2011b). Mince from dark muscle (100 g) was mixed with 300 ml of cold extracting buffer (10 mM Tris-HCl, pH 8.0 containing 1 mM EDTA and 25 g/l Triton X-100). The mixture was homogenized for 1 min at 13,000 rpm with Polytron Type PT 10/35 probe (Brinkmann Instruments, Westbury, NY, USA). Homogenate was subjected to centrifugation at 9,600*g* for 10 min at 4ºC using a Beckman J-6B centrifuge (Beckman Instruments Inc., Palo Alto, CA, USA). The supernatant obtained was filtered through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK). The filtrate, referred to as 'crude Mb extract', was subjected to ammonium sulphate (AS) fractionation (65-100% saturation). The precipitate obtained after centrifugation at 20,000*g* for 60 min was dissolved in a minimal volume of cold 5 mM Tris-HCl buffer, pH 8.5, which was referred to as 'starting buffer'. The mixture was then dialyzed against 10 volumes of the same buffer with 10 changes at 4ºC. The dialysate was immediately applied onto a Sephadex G-100 column (2.5 × 90 cm; Amersham Bioscience, Uppsala, Sweden), which was previously equilibrated with the starting buffer. The separation was conducted using the starting buffer at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and the absorbance at 280 and 540 nm was measured to monitor the proteins and heme proteins, respectively, using a UV-2401 spectrophotometer (Shimadzu Instruments, Inc., Columbia, MD, USA). The fractions with the high absorbance at 540 nm were pooled and referred to as 'Mb'. To locate Hb and Mb peaks, Hb from bighead carp blood was loaded onto a Sephadex G-100 column and the retention time was used to differentiate between Hb and Mb. Pooled Sephadex G-100 fractions containing Mb and Hb were determined by measuring the absorbance at 525 nm to quantify the Mb and Hb contents in the dark muscle of bighead carp, respectively. The molar extinction coefficient of 7.6×10^{-3} was used for the calculation (Tang *et al.*, 2004). Mb and Hb contents were expressed as mg/g dark mince.

7.3.3.2 Preparation of Hb

Hb was prepared as per the method of Fyhn *et al.* (1973). Four volumes of cold 1.7% NaCl in 1 mM Tris buffer (pH 8.0) were added to heparinized blood. The mixture was centrifuged at 700*g* for 10 min at 4ºC. After the plasma was removed, the red blood cells were

washed by suspending three times in 10 volumes of the same buffer. Red blood cells were lyzed in 3 volumes of 1 mM Tris (pH 8.0) for 1 h. One-tenth volume of 1 M NaCl was then added to aid in stromal removal before ultracentrifugation at 28,000*g* for 15 min at 4ºC using a Beckman L8-70M ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA, USA). Low molecular mass components (<10 kDa) were removed from 'Hb' using a prepacked PD-10 column (column volume: 13.5 ml, medium volume: 8.3 ml) (Bio-Rad, Hercules, CA, USA) eluted with 50 mM Tris buffer (pH 8.5) with the gravity flow.

7.3.3.3 Preparation of oxyMb/Hb and metMb/Hb

OxyMb/Hb and metMb/Hb were prepared according to the method of Tang *et al.* (2004) with some modifications. To obtain oxy-form, an aliquot (3 ml; 0.1 mM heme) of Mb or Hb solutions was added with 1.5 mg of sodium dithionite. MetMb/Hb was prepared by adding 1.5 mg of potassium ferricyanide to 3 ml of Mb or Hb solutions (0.1 mM). Sodium dithionite and potassium ferricyanide were removed by prepacked PD-10 columns and 20 mM phosphate buffer (pH 7.0) was used for elution with the gravity flow. At low pH (pH 6.0 of washed mince), Hb was susceptible to deoxygenation due to the Root effect. As a consequence, oxyHb was more likely a a mixture of oxy- and deoxyHb (data not shown) (Richards *et al.*, 2005).

7.3.3.4 Characterization of heme proteins

7.3.3.4.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Mb and Hb were subjected to SDS-PAGE according to the method of Laemmli (1970). Prestained protein marker (18-215 kDa) (Pierce, Rockford, IL, USA) was used for MW estimation.

7.3.3.4.2 Mass Spectrometry

A C4 zip-tip (Millipore, Billerica, MA, USA) was used to desalt Mb and Hb solutions. Desalted samples were analyzed by electrospray ionization mass spectrometry (ESI-MS) on an API365 Triple quadrupole (Applied Biosystems, Foster City, CA, USA). The ESI-MS raw spectra were obtained over the scan range from 600 to 1650 *m/z* and determined as a true mass scale (Richards *et al.*, 2005).

7.3.3.4.3 Determination of Mb and Hb concentration

The concentrations of Mb and Hb solutions, both oxy- and met-forms, were determined by measuring the absorbance at 525 nm. The molar extinction coefficient of 7.6×10^{-3} was used for the calculation and the concentration was expressed as μM heme protein.

7.3.3.4.4 Hemin loss from metMb and metHb

Hemin loss was determined as described by Grunwald and Richards (2006) using apoH64Y as a reagent to capture hemin from the met-forms of Mb and Hb. ApoH64Y was added at a 4-fold molar excess to that of the heme proteins (10 μM) on a monomer basis at pH 5.5 and 6.0. The mixtures were determined by measuring A_{600} and A_{700} during 4ºC storage. Binding of released hemin from heme proteins to the apoH64Y resulted in an increased A_{600}. Rate of hemin loss was reported as absorbance difference at wavelength of 600 nm and 700 nm (A_{600}-A_{700}).

7.3.4 Effect of oxyMb, metMb, oxyHb and metHb on lipid oxidation in washed mince

7.3.4.1 Preparation of washed mince

Washed mince of bighead carp was prepared following the method of Grunwald and Richards (2006) with a slight modification. The mince from light muscle was washed twice with cold distilled water with a mince/water ratio of 1:3 (w/v). The mixture was stirred continuously for 2 min with a glass rod and allowed to settle for 15 min. The mixture was filtered using 2 layers of cheese-cloth. Thereafter, 3 volumes of cold 50 mM sodium phosphate buffer (pH 6.0) were mixed with washed mince, stirred and filtered as described previously twice. The retentate was mixed with a designated buffer with a mince/buffer ratio of 1:1 (w/v) and homogenized for 3 min at a speed of 13,000 rpm. The pH of muscle slurry was checked and adjusted to the desirable pH (pH 6.0$\pm$0.05) using 0.5 M NaOH or 0.5 M HCl. After standing for 15 min, the mixture was centrifuged at 15,000*g* for 25 min. The pellet was collected and referred to as 'washed mince'. All processes were performed at 4ºC. Washed mince was packaged in polyethylene bag, vacuumed, sealed and stored at -80ºC.

7.3.4.2 Preparation of washed mince containing different forms of Mb and Hb

Washed mince was thawed overnight at 4ºC and ground until the homogeneity was obtained using a Hamilton Beach Custom Grind Type CM04 coffee grinder (Hamilton Beach/Proctor Silex, Inc., Southern Pines, NC, USA). The sample (20 g) was transferred to an

amber vial and added with phosphate buffer (pH 6.0) to obtain the final moisture content of 90%. In order to inhibit the microbial growth, streptomycin sulphate with a final concentration of 200 ppm was added to the washed mince. OxyMb, metMb, oxyHb or metHb was then added into washed mince to obtain a final concentration of 24 µmol heme/kg mince. For the control sample, phosphate buffer was added instead of heme protein solution. The mixtures in vial were mixed thoroughly with a plastic spatula. All samples were stored on ice for 9 days. Molten ice was removed and the same amount of ice was replaced every day. Samples were taken at day 0, 1, 2, 3, 5, 7 and 9 of storage for analyzes.

7.3.4.3 Analyzes

7.3.4.3.1 Measurement of Soret peak in heme proteins

To obtain the heme protein solution, washed mince (1g) was centrifuged at 14,000*g* for 10 min. The supernatants were then collected. Heme protein solutions were measured for absorption spectra ranging from 380 to 450 nm to monitor the Soret peak.

7.3.4.3.2 Measurement of redness

Colorimetric values of washed mince with different treatments were determined using a Minolta CR-300 Chroma Meter (Minolta Camera Co., Osaka, Japan). Washed mince samples were determined for *a*-value (redness/greenness).

7.3.4.3.3 Measurement of lipid peroxides

PV was determined as described by Richards and Hultin (2000) with some modifications. Washed mince (1 g) was mixed with 10 ml of a chloroform/methanol mixture (1:1, v/v). The mixture was homogenized at 13,500 rpm for 2 min and then added with 3.08 ml of 0.5% NaCl. The mixture was vortexed and centrifuged at 1,800*g* for 6 min. A 2 ml of the lower phase was added with 1.33 ml of cold chloroform/methanol (1:1) mixture, 25 µl of 30% (w/v) ammonium thiocyanate and 25 µl of 20 mM ferrous chloride in 3.5% HCl. The reaction mixture was allowed to stand for 20 min at room temperature and the absorbance at 500 nm was read. A standard curve was prepared using cumene hydroperoxide at concentrations ranging from 0 to 20 µM. PV was calculated and expressed as µmol cumene hydroperoxide/kg sample.

7.3.4.3.4 Measurement of thiobarbituric acid reactive substances (TBARS)

TBARS were determined following the method of Buege and Aust (1978). The sample (0.5 g) was homogenized with 2.5 ml of a solution containing 0.375% thiobarbituric acid

(w/v), 15% trichloroacetic acid (w/v) and 0.25 M HCl. The mixture was heated at 105ºC for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3,600*g* at 25ºC 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 40 µM. TBARS values were calculated and expressed as µmol malonaldehyde/kg sample.

7.3.4.3.5 Measurement of hexanal content

Hexanal content in washed mince with different treatments stored for 0, 3, 5 and 9 days was determined using a solid-phase micro-extraction (SPME) technique and gas chromatography (GC). The 10 ml-vial (Agilent Technologies, Palo Alto, CA, USA) with a metal cap and hole PTFE/silicone septa (MicroLiter Analytical Supplies Inc., Suwanee, GA, USA) containing 1 g of sample was allowed to stand at room temperature for 15 min, followed by heating at 40ºC for 5 min. The SPME fiber (PDMS/DVB, fused silica, d_f 65 µm, needle size 23 ga) (Supelco, Bellefonte, PA, USA) was conditioned at 260ºC for 30 min before use and then exposed to headspace of vial at 40ºC for 10 min.

GC analysis was performed using a HP 6890 gas chromatography (Hewlett-Packard, Palo Alto, CA, USA) equipped with capillary column (DB-5, 30 m length × 0.25 mm i.d. × 0.1 µm film thickness) and flame ionization detector (FID). After injection of SPME fiber into GC/FID injection port, hexanal from samples was desorbed from SPME fiber at 250ºC for 5 min. The flow rate of carrier gas was 1 ml/min. Inlet and detector temperatures were 250 and 270ºC, respectively. The oven temperature was programmed at 40ºC for 5 min with a 10ºC/min ramp rate until 90ºC. Identification of hexanal was based on the retention time. Quantification of hexanal in the sample was made using a standard curve of hexanal solution (0-0.25 ppm).

7.3.5 Statistical analysis

Data from triplicate run were subjected to analysis of variance (ANOVA) with the confidence level of 95%. Comparison of means was carried out by Duncan's multiple range test (Steel and Torrie, 1980). For pair comparison, T-test was used. Statistical analysis was performed using the Statistical Package for Social Science (SPSS 14.0 for windows, SPSS Inc., Chicago, IL, USA).

7.4 Results and Discussion

7.4.1 Characteristics of Mb and Hb from bighead carp

7.4.1.1 Purification of Mb and Hb

Heme proteins of bighead carp dark muscle were firstly extracted. Thereafter, the pellet precipitated using AS was chromatographed using a Sephadex G-100 column and two major peaks of A_{540} were obtained (Figure 30A).

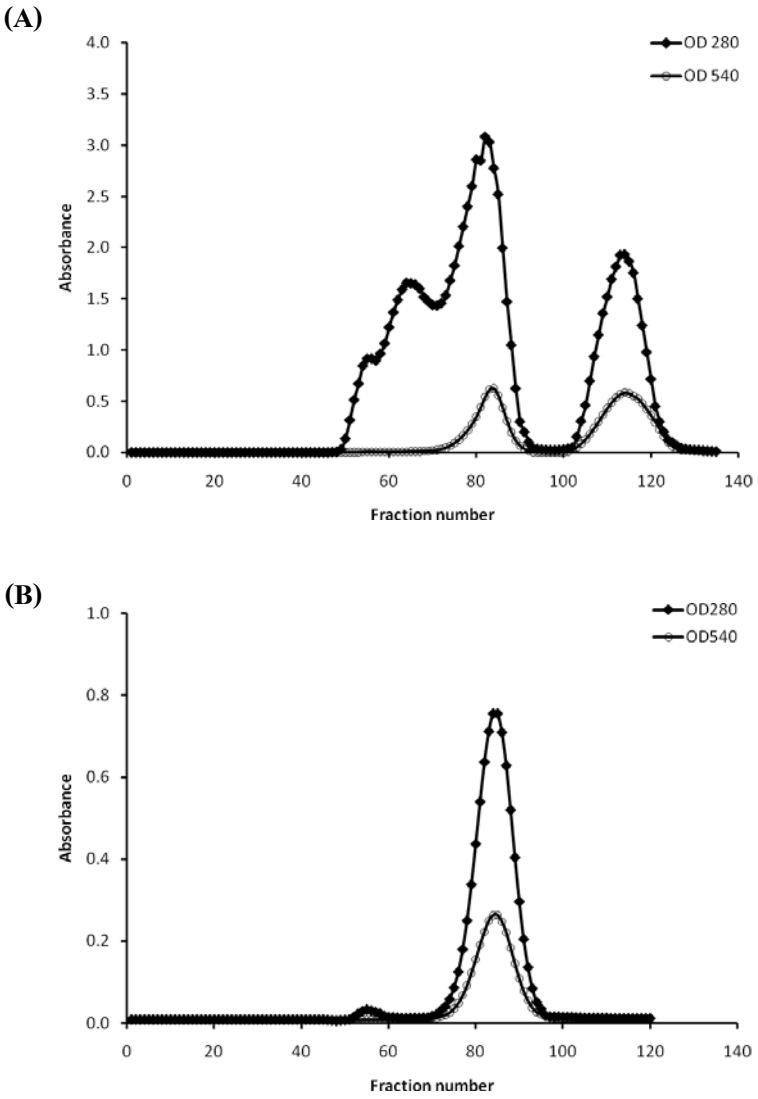


Figure 30. Elution profile of Mb purified from bighead carp dark muscle (A) and Hb from bighead carp blood (B) on Sephadex G-100

Pooled fractions of the first peak having red color more likely contained Hb as the major component, whereas pooled fractions of the second peak with brown color were presumed to contain Mb. The two heme proteins, Mb and Hb, could be distinguished on the basis of different size. In general, Hb has 4-fold higher MW than does Mb. Hb peak in the bighead carp dark muscle extract was confirmed with elution profile of Hb from bighead carp blood on Sephadex G-100 column, in which the same retention time was observed (Figure 30B). Mb and Hb contents in the dark muscle of bighead carp were determined to be 1.36 and 0.86 mg/g dark mince, respectively. Brown (1962) reported that dark muscle of yellowfin tuna (*Neothunnus macropterus*) contained Mb ranging from 5.3 to 24.4 mg/g, while Hb ranged from 0.5 to 3.8 mg/g.

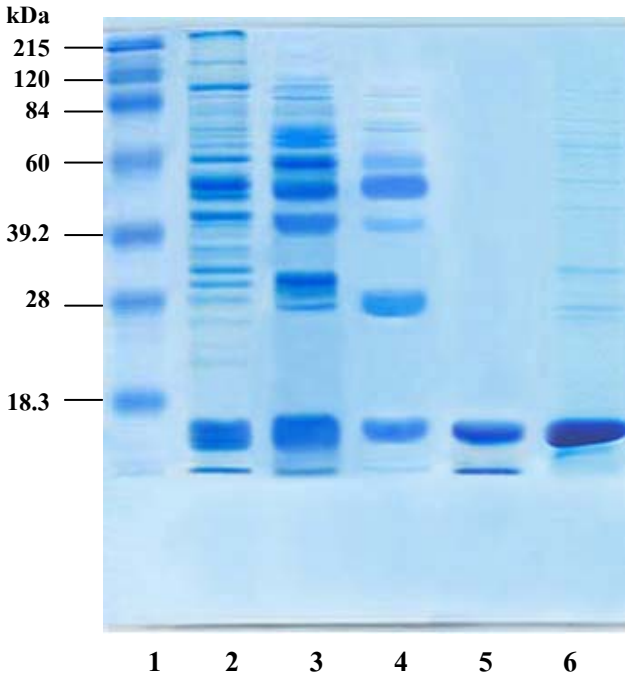


Figure 31. SDS-PAGE pattern of crude extract and different fractions containing Mb from bighead carp dark muscle and Hb from bighead carp blood; lane 1, MW marker; lane 2, crude extract from bighead carp dark muscle; lane 3, AS fraction; lane 4, pooled Sephadex G-100 fraction I (No. 72-90); lane 5, pooled Sephadex G-100 fraction II (No. 114-126); lane 6, Hb from bighead carp blood

Protein patterns of extract, AS fraction and Sephadex G-100 fraction from bighead carp dark muscle and Hb from bighead carp blood are shown in Figure 31. Crude extract contained proteins with MW ranging from 16 to 120 kDa (Figure 31, lane 2), while AS fraction consisted of proteins with MW range of 16-90 kDa (Figure 31, lane 3). Different protein patterns were observed between both samples. It was noted that protein with MW of ~16 kDa was dominant in both samples. The first peak obtained from Sephadex G-100 column (fraction No. 72-90) comprised several proteins and those with MW of 50, 28 and ~16 kDa were dominant (Figure 31, lane 4). The second peak (fraction No. 114-126) showed the predominant band with MW of ~16 kDa, which was likely Mb (Figure 31, lane 5). The densitometric analysis indicated that Mb represented 89% of total protein in pooled fractions of the second peak. This suggested an effective removal of contaminating proteins in crude extract from bighead carp dark muscle after being precipitated with AS (65-100% saturation) and chromatographed with Sephadex G-100 gel filtration. Protein pattern of Hb prepared from bighead carp blood exhibited the intense band at MW of ~16 kDa (Figure 31, lane 6) indicating that Hb was the main protein in red blood cell. Under the reducing condition of SDS-PAGE, a tetramer of Hb was dissociated into a monomer with MW of 16 kDa.

Molecular mass of Mb from the dark muscle of bighead carp and Hb from the blood were also determined by ESI-MS (Figure 32). For Mb enriched Sephadex G-100 fractions (the second peak), a major peak at 15,829 Da was obtained, likely representing the globin of bighead carp Mb. Two minor peaks at 15,863 and 15,927 Da were also noticeable. Mb consists of a globin and the heme group with a MW of 616 Da. During ionisation, Mb is dissociated into the globin and heme moieties and they are detected as the separated entities (Richards *et al.*, 2005). For Hb extracted from the blood of adult bighead carp, the predominant peak at 15,390 Da was observed, representing α-chain of Hb. Two additional peaks obtained at 15,488 and 15,564 Da represented two β-chains of Hb. Hb from adult and juvenile bighead carp were similar except that the juvenile Hb had no additional β-chain II of 15,564 Da (data not shown). Hilse and Braunitzer (1968) reported that Hb α-chain of common carp (*Cyprinus carpio*) had 142 amino acids with MW of 15,248 Da.

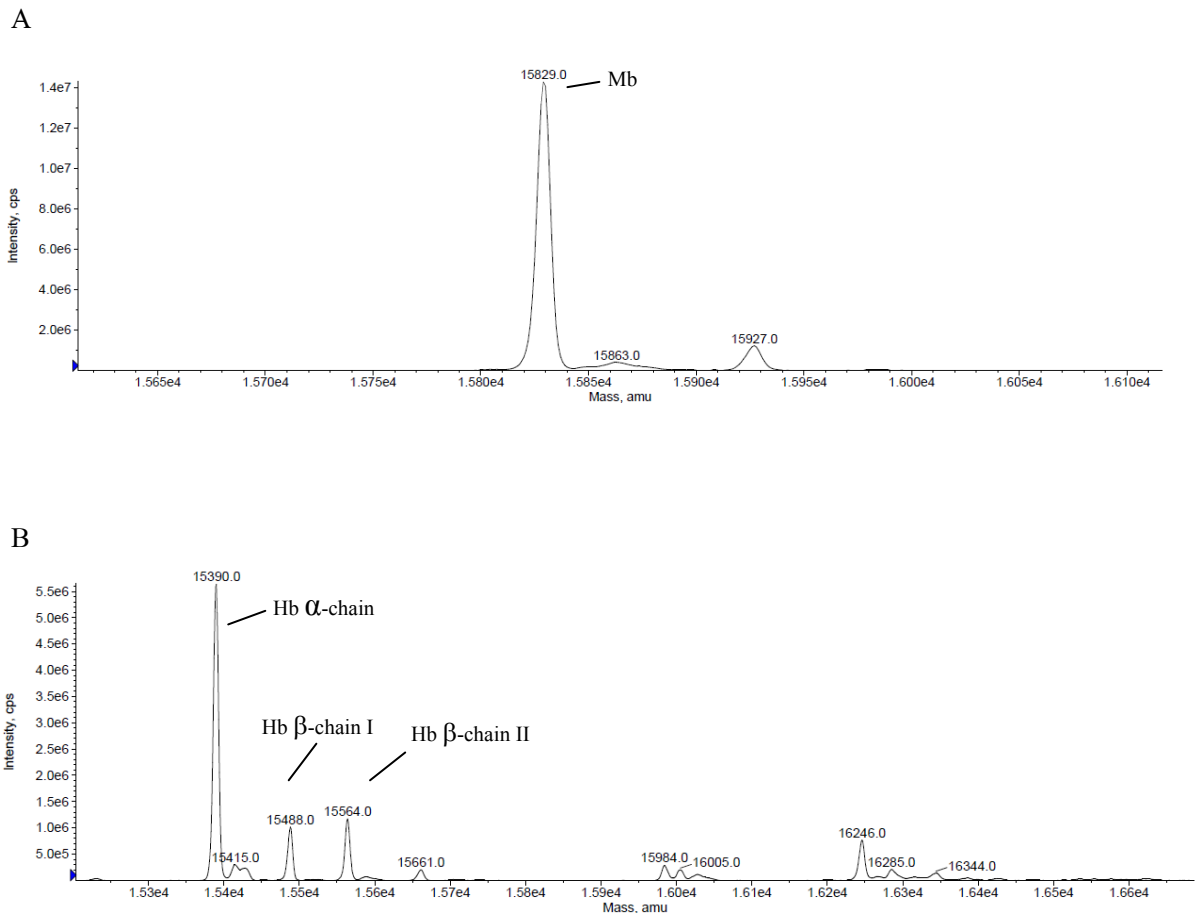


Figure 32. ESI-MS of Mb from bighead carp dark muscle in pooled Sephadex G-100 fraction II (A) and Hb from adult bighead carp blood (B)

7.4.1.2 Hemin loss from metMb and metHb

Hemin loss from metMb and metHb was examined at pH 6.0 during 4ºC storage (Figure 33A). ApoH64Y with the tyrosine substitution has affinity for hemin (Hargrove *et al.*, 1994). Hemin released from Mb or Hb was gathered by this apoglobin and specific chromophore was generated (Grunwald and Richards, 2006; Hargrove *et al.*, 1994). High value of A_{600}-A_{700} was found in metHb sample, indicating that hemin loss from metHb was rapid at pH 6.0. Conversely, a little hemin loss from metMb was observed. The result indicated that a higher hemin loss took place in metHb, compared to metMb at pH 6.0. Thus, metHb had the lower hemin affinity than metMb. For Mb, the highly conserved histidine at site FG3 and prevalent serine or threonine at F7 stabilize the heme moiety within the globin while Hb contain leucine

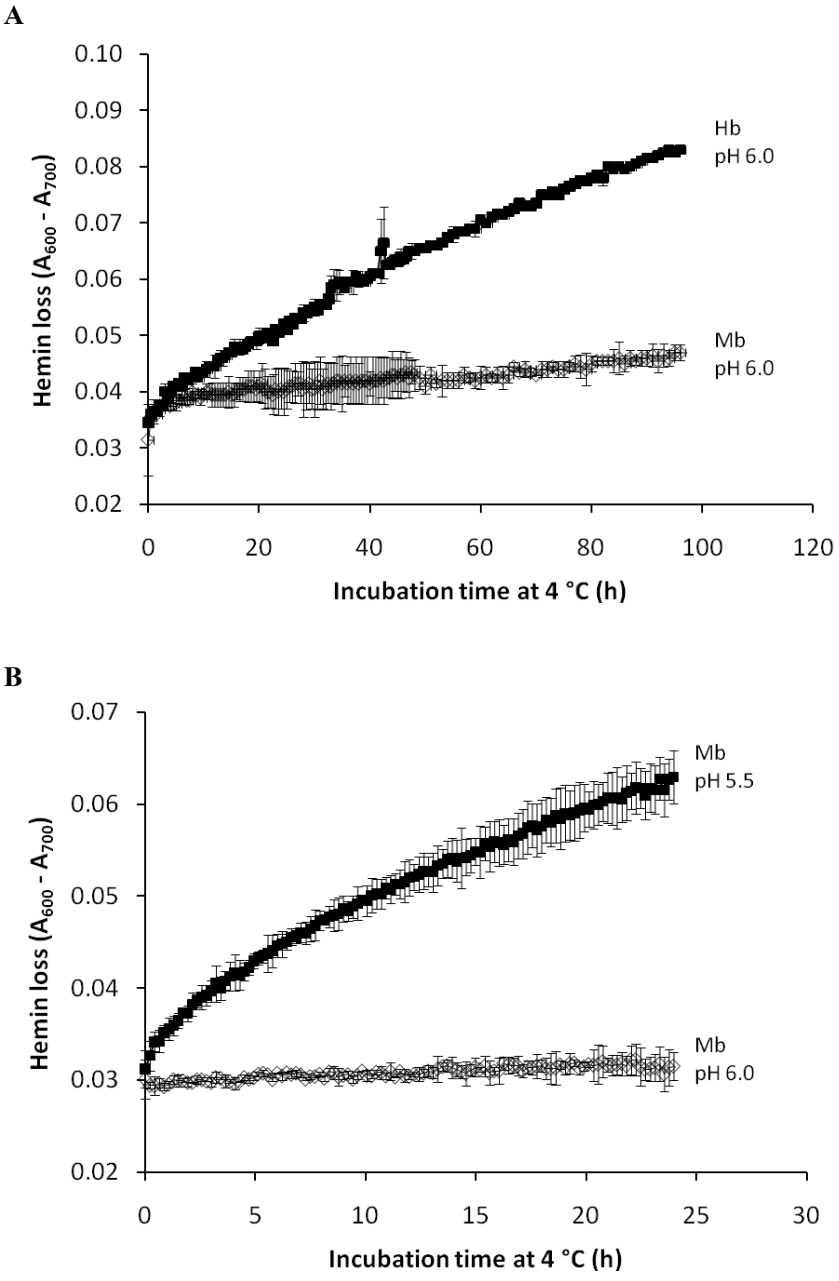


Figure 33. Hemin loss (A_{600}-A_{700}) from bighead carp metMb and metHb at pH 6.0 (A) and metMb at pH 5.5 and pH 6.0 (B) using apoH64Y as hemin loss reagent during storage at 4ºC. Bars represent standard deviation (n=3)

residues at these sites (Richards, 2010). Hemin loss from metMb increased rapidly when pH decreased from 6.0 to 5.5 (Figure 33B). At acidic pH, an enhanced protonation of Mb induced conformational changes, as well as the unfolding of globin which could reduce the stability of heme-globin linkage (Thiansilakul *et al.*, 2011b). Sperm whale metMb had a 20-fold lower hemin affinity at pH 5.5, compared to pH 6.0 and 200-fold lower at pH 5.0, compared to pH 6.0 (Hargrove *et al.*, 1994). Thiansilakul *et al.* (2011b) suggested a degradation of the heme structure of tuna oxy- and metMb at pH less than 5.0. Thus, composition and conformation of globin greatly influenced hemin affinity in heme proteins.

7.4.2 Effect of oxyMb, metMb, oxyHb and metHb on lipid oxidation in washed mince during iced storage

7.4.2.1 Changes in Soret peak of the supernatant after centrifugation of washed mince

The Soret wavelengths of Mb and Hb with different forms in the supernatant after centrifugation of washed mince are shown in Figure 34. At day 0, oxyMb, oxyHb, metMb and metHb samples had the Soret peak at wavelengths of 416, 412, 406 and 406 nm, respectively. Mb from Eastern little tuna had the Soret peak at wavelength of 413 for oxy-form and 407 nm for met-form (Thiansilakul *et al.*, 2011b). The Soret peak of oxy-heme proteins had the higher wavelength than that of met-heme proteins. The Soret peak of oxyMb sample gradually decreased to the wavelength of 410 nm when storage time increased up to day 9. In oxyHb sample, the wavelength of the Soret peak sharply decreased to 404 nm at day 2 of storage and increased to 406 nm at day 5. Thereafter, the Soret peak remained constant at wavelength of 406 nm until day 9 of storage. The Soret absorbance of metMb is reported to range from 406 to 410 nm (Smulevich *et al.*, 2007; Swatland, 1989; Thiansilakul *et al.*, 2011b). The reported Soret absorbance for metHb is 405 nm (Antonini and Brunori, 1971). FerrylMb is noted for having an extinction coefficient at 420 nm (Rao *et al.*, 1994) and crosslinking of Mb after ferrylMb formation is observed at 405 nm (Osawa and Williams, 1996). Changes in the Soret wavelength (416 to 410 nm) suggested that oxyMb samples were gradually converted to metMb with the possibility of some ferrylMb and cross-linked Mb being present simultaneously. Nearly all the oxyMb remained intact as holoMb during the 9 days of storage based on constant and elevated Soret

absorbances (data not shown). For oxyHb, a sharp decrease in the Soret wavelength indicated the susceptibility of oxyHb to autoxidation. The Soret wavelength for the oxyHb sample was between 404 and 406 nm from day 2 to 9 which could be detection of metHb or a combination of metHb and a cross-linked Hb specie. Approximately one-fourth of the oxyHb sample remained as holoHb based on changes in Soret absorbance during the 9 days of storage (data not shown).

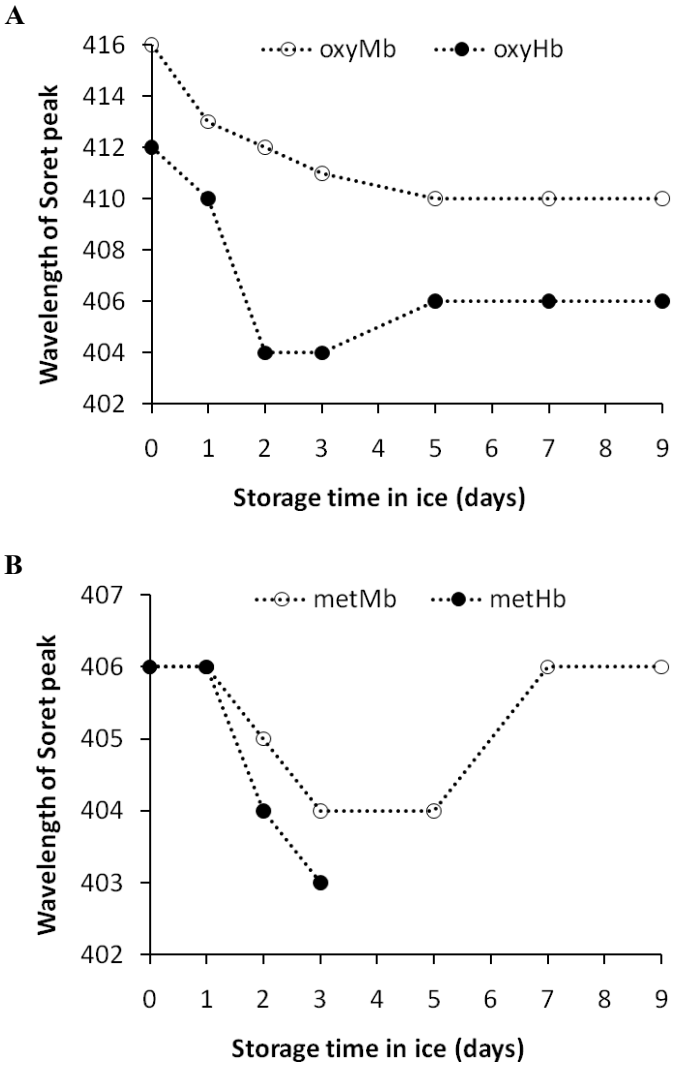


Figure 34. Changes in wavelength of the Soret peak in supernatants of washed mince containing oxyMb and oxyHb (A) and metMb and metHb (B) from bighead carp during 9 days of iced storage, pH 6.0

In washed mince containing metMb, the Soret peak decreased to 404 nm during 3-5 days of storage and then increased to 406 nm during 7-9 days of storage suggesting the possible formation of ferrylMb and cross-linked Mb during storage. The shift of the Soret wavelength was similar to that of oxyHb sample. The cycling of ferrylMb and metMb can occur during storage, leading to a difficultly to directly measure ferrylMb. Also, metMb tended to remain as holoMb during storage based on constant and elevated Soret absorbances (data not shown). In metHb sample, the wavelength of the Soret peak was observed at 406 nm at day 1 and then decreased to 403 nm by day 3 of storage. Thereafter, no Soret peak was detectable after 3 days of storage, reflecting the disappearance of heme protein. A Soret band results mainly from the interaction of the heme moiety with globin. Hence it can be used to monitor the unfolding of hemoproteins (Thiansilakul *et al.*, 2011b). Degradation of heme protein found in metHb sample was coincidental with rapid hemin loss from metHb at pH 6.0. Weak heme-globin linkage as well as rapid autoxidation of Hb revealed the lower stability of Hb than Mb.

7.4.2.2 Changes in redness

Redness (*a*-value) of washed mince without and with the addition of varying heme proteins during iced storage is shown in Figure 35. Washed mince without the addition of heme protein had low *a*-values throughout 9 days of storage. The result indicated the efficiency of washing process in removal of water soluble pigments from bighead carp muscle. At day 0, washed mince containing oxyMb exhibited the highest redness, followed by those added with oxyHb, metMb, metHb and the control, respectively ($P < 0.05$). Mb and Hb, the heme proteins, are the major pigments found in fish, especially in the dark muscle. Heme protein in the oxy-form is bright red, which is considered for the fresh and attractive meat. On the other hand, heme protein with the met-form is brown and rather unattractive (Faustman and Cassens, 1990). During 9 days of iced storage, the gradual decreases in redness of washed mince containing oxyMb and metMb were noticeable ($P < 0.05$). It was noted that washed mince containing oxyMb had a higher redness than that having metMb throughout the storage ($P < 0.05$). For washed mince containing oxyHb and metHb, sharp decreases in redness were observed within the first 3 days of storage ($P < 0.05$). Thereafter the redness tended to be stable and showed the slight difference between both samples. After 3 days of storage, the lowest redness of washed mince containing

oxyHb and metHb were observed, compared with other samples until the end of storage ($P < 0.05$).

The relative amounts of the oxidation states of heme proteins in muscle contribute to the apparent color (Faustman and Cassens, 1990; Tang *et al.*, 2004). Mb or Hb in the oxy-form can be oxidized during storage, resulting in discoloration of washed mince. A rapid decrease in redness of washed mince containing oxyHb in the early stage of storage indicated the susceptibility of oxyHb to autoxidation, compared with oxyMb. Decrease in *a*-value was more rapid and extensive in washed mince containing metHb, compared to that added with metMb. This was likely due to the higher dissociation of hemin from metHb, while negligible hemin dissociation occurred in metMb (Figure 33). Thus, changes in color of washed mince containing Mb or Hb could be governed by the stability of heme proteins toward autoxidation as well as heme dissociation from globin.

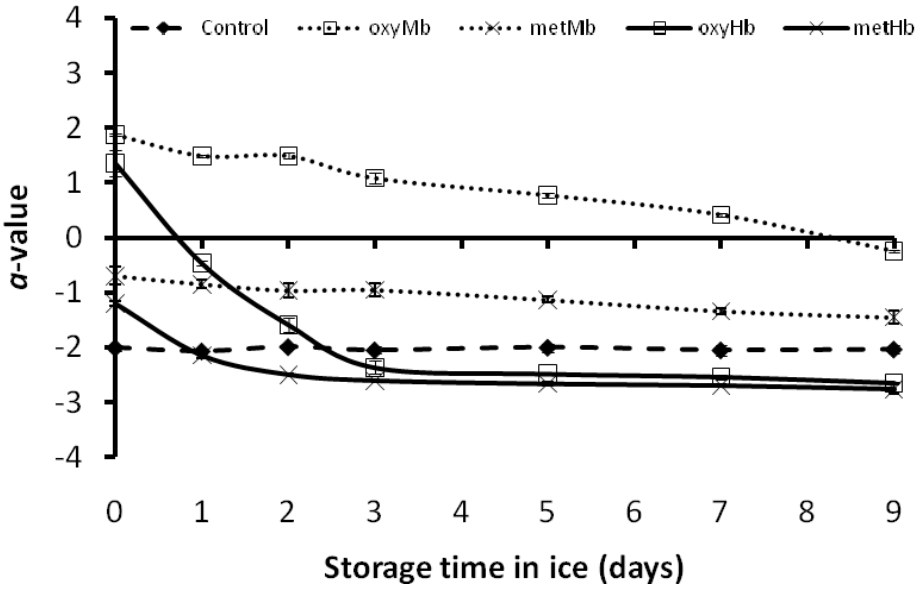


Figure 35. Changes in redness (*a*-value) of washed mince added with oxyMb, metMb, oxyHb and metHb from bighead carp during 9 days of iced storage, pH 6.0. Bars represent standard deviation (n=3)

7.4.2.3 Changes in lipid peroxides

Lipid peroxides (PV) in washed mince without and with the addition of oxyMb, metMb, oxyHb and metHb during 9 days of iced storage is depicted in Figure 36A. Washed mince without heme proteins (the control) showed slight increase in PV as the storage time increased ($P < 0.05$). The lowest PV was noticed in the control, compared with those containing heme proteins throughout 9 days of storage ($P < 0.05$). Washed mince containing oxyMb and metMb had a gradual increase in PV as the storage time increased. When oxyHb and metHb were incorporated, washed mince exhibited rapid and extensive increases in PV up to 5 days of storage, followed by a slight decrease until the end of storage ($P < 0.05$). The results indicated that Hb in both oxy- and met-forms were much more capable of promoting lipid peroxide formation than did Mb. One possible mechanism of rapid lipid oxidation induced by Hb was the greater hemin loss from Hb compared to Mb (Figure 33). Hemin loss was also found to be more rapid in trout Hb, compared to trout Mb at pH 6.3 (Richards *et al.*, 2005). A rapid hemin loss from metHb facilitated lipid oxidation in washed mince, whereas metMb with the retained porphyrin was less reactive for accelerating lipid oxidation. Recombinant heme proteins had higher ability to promote lipid oxidation in washed mince, compared to heme proteins that had higher hemin affinity (Grunwald and Richards, 2006).

Within the first 2 days of iced storage, washed mince containing metHb had higher PV than that added with oxyHb. Nevertheless, higher PV was observed in washed mince containing oxyHb during 3-9 days of storage. MetHb promoted lipid oxidation more rapidly than did oxyHb in the early stage of storage. The potent pro-oxidative activity of metHb could be attributed to its hemin loss. Essentially negligible rates of hemin loss were reported for oxyHb and deoxyHb, whereas extensive hemin release was observed for metHb (Bunn and Jandl, 1966). Lipid oxidation in washed mince containing oxyHb was associated with superoxide radicals generated during autoxidation (Richards and Dettmann, 2003). Superoxide radicals can be converted to H_2O_2 that facilitate ferrylHb catalyst formation (Kanner and Harel, 1985) and increase the reactivity of dissociated hemin (Robinson *et al.*, 2009). OxyHb, on the other hand, is gradually oxidized to metHb, which is more prone to hemin loss. When comparing pro-oxidative activity between both forms of Mb, higher PV was found in washed mince containing metMb,

compared with that having oxyMb throughout storage ($P < 0.05$). MetMb was able to generate ferrylMb and cross-linked Mb, which could promote lipid oxidation (Vuletich *et al.*, 2000).

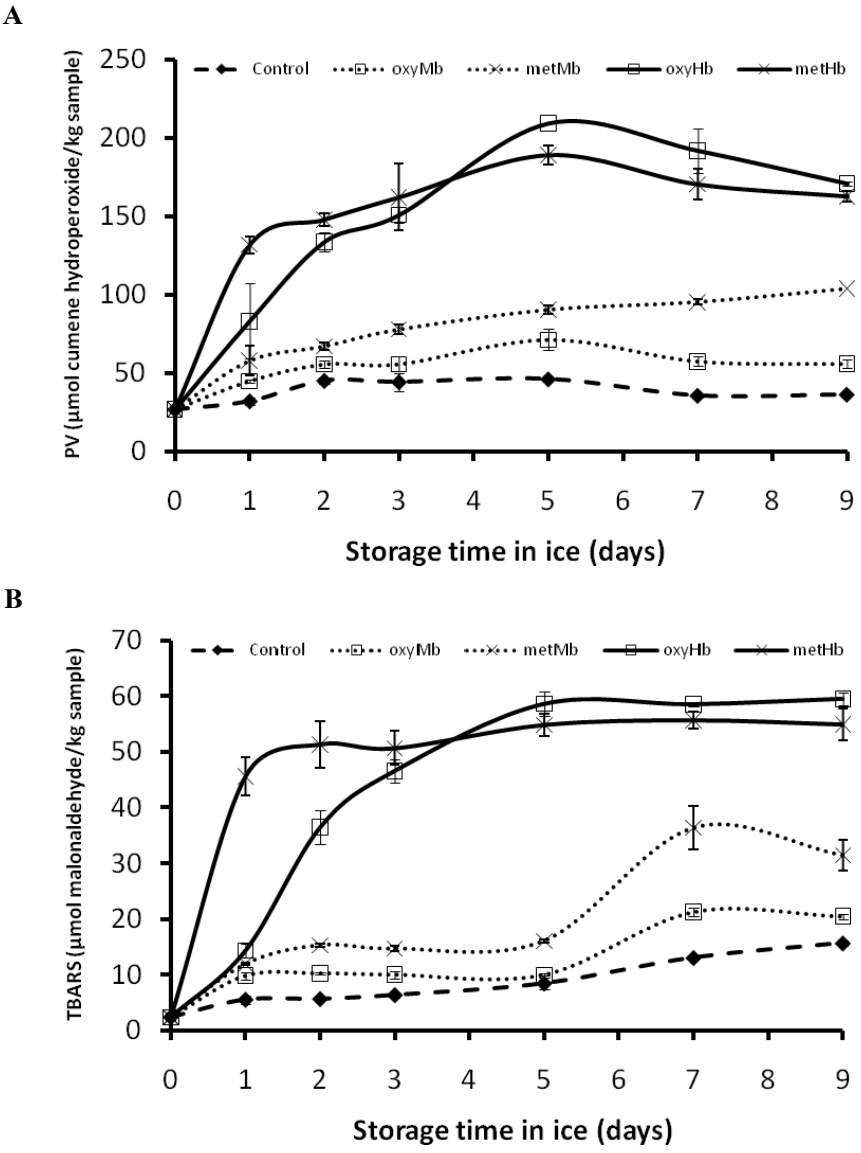


Figure 36. Changes in PV (A) and TBARS (B) values of washed mince added with oxyMb, metMb, oxyHb and metHb from bighead carp during 9 days of iced storage, pH 6.0. Bars represent standard deviation (n=3)

7.4.2.4 Changes in TBARS

Changes in TBARS of washed mince without and with the addition of varying heme proteins during iced storage are shown in Figure 36B. In the presence of heme proteins, the increases in TBARS were observed, suggesting the effective pro-oxidative activity of heme proteins in washed mince. For washed mince having oxyMb and metMb, a gradual increase in TBARS was noticed during the first 5 days of storage, followed by a sharper increase at day 7 of storage ($P < 0.05$). A rapid increase in TBARS was found in washed mince containing oxyHb within the first 5 days of storage and no change in TBARS was observed thereafter ($P > 0.05$). For washed mince containing metHb, TBARS increased dramatically within the first 2 days of iced storage. No changes in TBARS were observed during 3-9 days of storage ($P > 0.05$). Washed mince with the addition of oxyHb and metHb showed more rapid and extensive TBARS formation than those having oxyMb and metMb. Low hemin release from metMb should be considered as part of the mechanism by which lipid oxidation was accelerated. In addition, H_2O_2 equivalents could be formed during Hb oxidation, which rapidly occurred compared to Mb oxidation. Thus, the formation of ferrylHb could be a part of the mechanism by which bighead carp Hb promoted lipid oxidation in washed mince. However, the strong pigment bleaching in washed mince containing oxy- or metHb during storage (Figure 35) indicated the more important role of hemin loss from Hb to initiate lipid oxidation compared to a role of ferrylHb catalyst. Monahan *et al.* (1993) reported that lipid oxidation was highest in washed pork muscle treated with Hb, followed by Mb and non-heme iron, respectively.

Based on TBARS in washed mince, metMb had a higher effect on acceleration of lipid oxidation in washed mince when compared with oxyMb throughout the storage ($P < 0.05$). MetMb can react with H_2O_2 or pre-formed lipid hydroperoxides to form ferrylMb catalyst that can stimulate lipid oxidation (Kanner and Harel, 1985). The slow formation of metMb from oxyMb during storage explains the relatively slow formation of TBARS in the oxyMb samples.

7.4.2.5 Changes in hexanal content

Hexanal contents of washed mince added with and without heme proteins during 9 days of iced storage are shown in Figure 37. Washed mince without heme protein added had the lowest hexanal content, in comparison with the other samples throughout 9 days of storage ($P < 0.05$). During storage, the highest formation of hexanal was observed in washed mince

incorporated with metHb, followed by oxyHb, metMb and oxyMb, respectively. However, at day 5 of storage, higher hexanal content was observed in washed mince containing oxyHb than that having metHb ($P < 0.05$). Hexanal contents in all samples gradually decreased at day 9 of storage ($P < 0.05$). Oxidation of lipids is a major cause of deterioration of fish and fish products, especially those containing high content of unsaturated fatty acids. Several derivatives of aldehyde, ketone and alcohol could be formed via lipid oxidation in washed silver carp induced by Hb (Fu *et al.*, 2009) and washed Asian seabass added with Mb (Thiansilakul *et al.*, 2011a). Hexanal generated during storage time would contribute to off-odor and fishy odor development in bighead carp muscle. The result also reconfirmed a higher impact of Hb than Mb on lipid oxidation, especially when met-form was present. Roles of dissociated hemin, ferryl-heme protein and cross-linked heme protein in lipid oxidation should be further examined to elucidate the specific mechanisms of heme-protein mediated lipid oxidation.

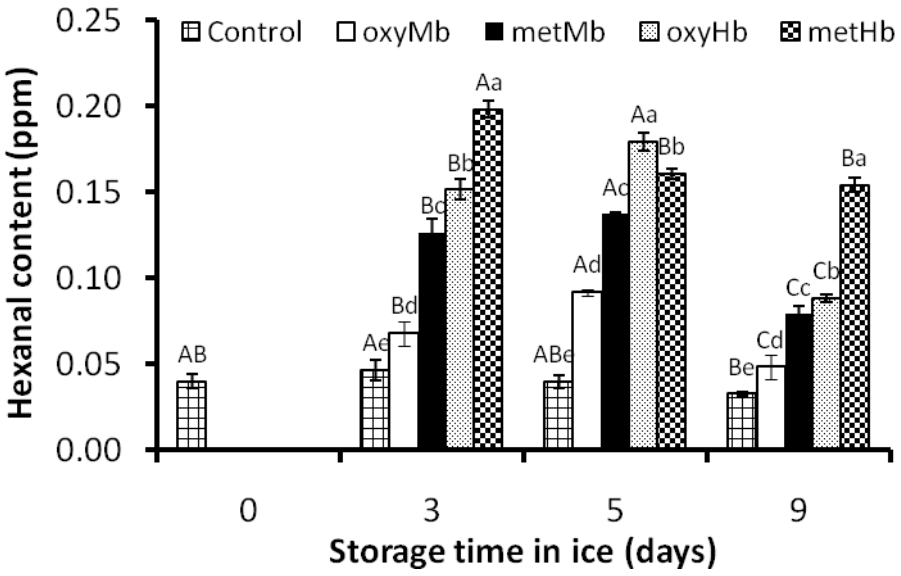


Figure 37. Changes in hexanal content of washed mince added with oxyMb, metMb, oxyHb and metHb from bighead carp during 9 days of iced storage, pH 6.0. Bars represent standard deviation (n=3)

7.5 Conclusions

Mb and Hb were prepared from the dark muscle and blood of bighead carp, respectively. Mb and Hb monomer had MW of ~16 kDa. During 9 days of iced storage, Hb showed a higher negative impact on color of washed mince in comparison with Mb. In the presence of heme proteins, lipid oxidation of washed mince occurred to a greater extent during storage time. Both Mb and Hb could accelerate lipid oxidation, in which primary and secondary oxidation products were formed. However, Hb which had a rapid hemin loss was more pro-oxidative than Mb. Decreasing the concentration of both heme proteins, especially Hb, could be an approach to retard lipid oxidation in fish and fish products so that quality can be maintained.

CHAPTER 8

Retardation of myoglobin and hemoglobin-mediated lipid oxidation in washed bighead carp by phenolic compounds

8.1 Abstract

Antioxidative activities of different phenolic compounds (caffeic acid, gallic acid and tannic acid) at a level of 200 ppm in washed mince added with myoglobin (Mb) and hemoglobin (Hb) from bighead carp (*Hypophthalmichthys nobilis*) at pH 6 during 9 days of iced storage were studied. Tannic acid exhibited the preventive effect on discoloration of washed mince containing Mb or Hb ($P < 0.05$), whereas caffeic acid and gallic acid were not able to maintain redness of washed mince during storage. Peroxide value (PV), thiobarbituric acid-reactive substances (TBARS) and hexanal were greatly formed in washed mince containing heme proteins, especially Hb. As determined by apo Streptococcal heme-associated protein (apoShp), heme release from Hb into apoShp was higher than that from Mb, suggesting the lower heme stability and higher pro-oxidative activity of Hb than Mb. Phenolic compounds could lower lipid oxidation induced by Mb or Hb throughout storage ($P < 0.05$). Caffeic acid and gallic acid generally showed the higher antioxidative activities than did tannic acid, especially at the extended storage time ($P < 0.05$). Prevention of heme release as well as inhibition of lipid oxidation induced by heme proteins with selected phenolic compound should be an alternative means in lowering discoloration and lipid oxidation in post-mortem fish.

8.2 Introduction

Myoglobin (Mb) and hemoglobin (Hb) are the most abundant heme proteins found *in vivo* and are primarily related to meat color. Heme protein comprises polypeptide chain called 'globin' and iron protoporphyrin-IX complex called 'heme'. Mb, a monomeric heme protein, is mainly localized in muscle tissue and serves as an oxygen storage site (Faustman and Cassens, 1990). Hb, a tetrameric heme protein, is highly concentrated in the erythrocytes and responsible for transporting oxygen (Jensen, 2004; Stryer, 1988). However, blood capillaries can

burst naturally and also during mechanical treatments such as filleting and mincing, leading to the distribution of Hb throughout the muscle.

Heme proteins have a close relationship with lipid oxidation, which negatively affects the quality of muscle food. Immediately after death, Mb and Hb are mostly present in the reduced form or the ferrous state (Mb/Hb-Fe^{2+}), which provides the attractive red color to the muscle. With increasing storage time, the iron atom in the heme ring is oxidized to the ferric state (Mb/Hb-Fe^{3+}), which is responsible for the discoloration of meat and acceleration of lipid oxidation (Baron and Andersen, 2002; Lee *et al.*, 2003). Oxidation of Mb and Hb played a considerable role in the formation of hydroperoxides and promoted lipid oxidation in fish muscle (Richards *et al.*, 2005; Thiansilakul *et al.*, 2011a). Interaction between met-heme protein with H_2O_2 results in the generation of ferryl-heme protein (Mb/Hb-Fe^{4+}), a strong pro-oxidant (Baron and Andersen, 2002). Ferryl-heme protein can abstract hydrogen atom from polyunsaturated fatty acids and hence initiate lipid oxidation (Galaris *et al.*, 1990). Grunwald and Richards (2006) revealed that heme loss rate of heme protein was more crucial on lipid oxidation as compared to autoxidation rate.

To retard the development of rancidity and other deteriorations associated with lipid oxidation in muscle foods, antioxidants are used to scavenge free radicals and terminate free radical chain propagation (Medina *et al.*, 2007; Nawar, 1996; Pazos *et al.*, 2006). Phenolic compounds, the bioactive substances widely distributed in plants, such as tea catechins, grape procyanidins, rosemary extracts and olive oil hydroxytyrosol have been reported for their antioxidative activity in fish and fish products (Pazos *et al.*, 2006; Tang *et al.*, 2001; Vareltzis *et al.*, 1997). Phenolic compounds vary in structure and number of hydroxyl groups (Figure 38), with varying antioxidative property (Medina *et al.*, 2007). These compounds have been used for inhibition of lipid oxidation as well as improving overall quality of fish or fish product, especially those containing high content of heme pigments (Medina *et al.*, 2007; Tang *et al.*, 2001). The objective of this investigation was to elucidate the impact of different phenolic compounds on prevention of discoloration and lipid oxidation in washed bighead carp added with Mb and Hb during iced storage.

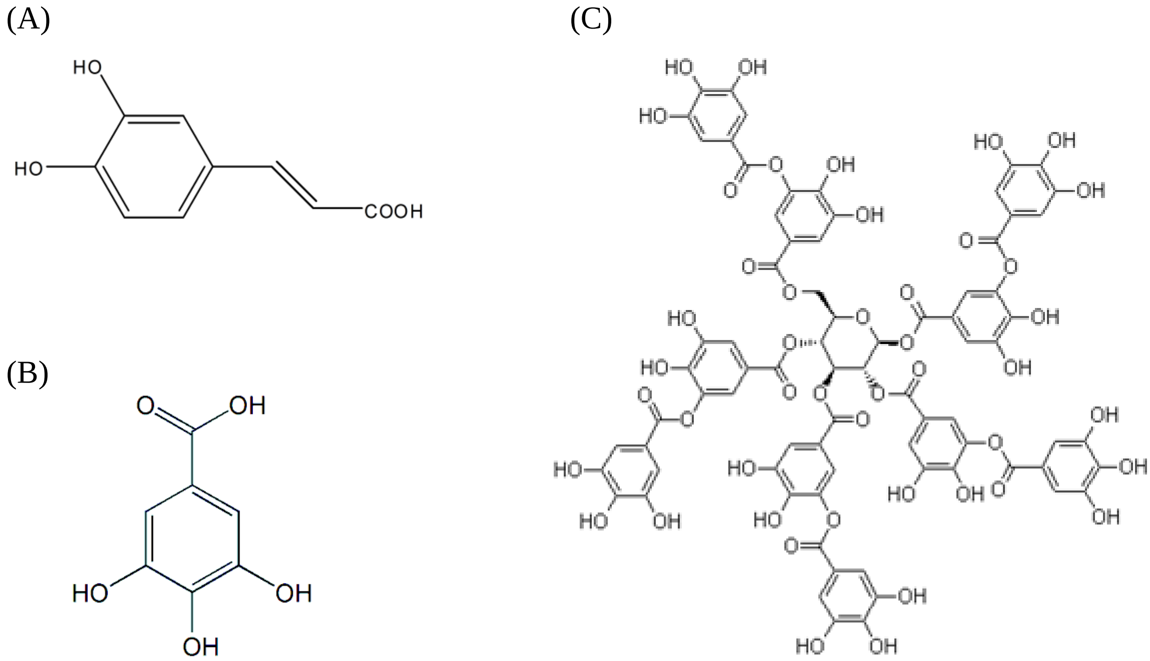


Figure 38. Structures of three phenolic compounds; caffeic acid (A), gallic acid (B) and tannic acid (C)

8.3 Materials and methods

8.3.1 Chemicals

Triton X-100, sodium dithionite, ferrous chloride, chloroform, methanol, agar, tryptone and yeast extract were procured from Fisher Scientific (Pittsburgh, PA, USA). Streptomycin sulfate, ammonium thiocyanate, 2-thiobarbituric acid, cumene hydroperoxide, 1,1,3,3-tetramethoxypropane, caffeic acid, gallic acid and tannic acid were purchased from Sigma (St. Louis, MO, USA). Isopropyl-D-thiogalactopyranoside (IPTG) was obtained from Promega (Madison, WI, USA). All chemicals were of analytical grade.

8.3.2 Collection of fish samples and blood

Blood and fillets from adult bighead carp (50-60 cm in length) were obtained from Schafer Fisheries (Thomson, IL, USA). Blood was removed from the caudal vein by severing the caudal peduncal and collecting blood in salined sodium heparin (30 Units heparin per ml of blood drawn). The fish fillets and heparinized blood were placed in an insulated box containing frozen gel packs and transported to the Meat Science and Muscle Biology Laboratory, University of Wisconsin-Madison (Madison, WI, USA) within 1 day after capture. Upon arrival, dark and ordinary muscles were separated and cut into small pieces. Each samples were ground using a KSM90WW household mixer (Kitchen Aid, Inc., St. Joseph, MI, USA) until uniformity was obtained. Mince from dark and ordinary muscle was used for preparation of Mb and washed mince, respectively, whereas heparinized blood was used for Hb preparation.

8.3.3 Preparation of Mb and Hb from bighead carp

8.3.3.1 Preparation of Mb

Extraction and purification of Mb were performed according to the method of Thiansilakul *et al.* (2011b). Mince from dark muscle (100 g) was mixed with 300 ml of cold extracting buffer (10 mM Tris-HCl, pH 8.0 containing 1 mM EDTA and 25 g/l Triton X-100). The mixture was homogenized for 1 min at 13,000 rpm with Polytron Type PT 10/35 probe (Brinkmann Instruments, Westbury, NY, USA). Homogenate was subjected to centrifugation at 9,600*g* for 10 min at 4ºC using a Beckman J-6B centrifuge (Beckman Instruments Inc., Palo Alto, CA, USA). The supernatant obtained was filtered through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK). The filtrate, referred to as 'crude Mb extract', was subjected to ammonium sulfate fractionation (65-100% saturation). The precipitate obtained after centrifugation at 20,000*g* for 60 min was dissolved in a minimal volume of cold 5 mM Tris-HCl buffer, pH 8.5, which was referred to as 'starting buffer'. The mixture was then dialyzed against 10 volumes of the same buffer with 10 changes at 4ºC. The dialysate was immediately applied onto a Sephadex G-100 column (2.5 × 90 cm; Amersham Bioscience, Uppsala, Sweden), which was previously equilibrated with the starting buffer. The separation was conducted using the starting buffer at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and the absorbance at 280 and 540 nm was measured to monitor the proteins and heme proteins, respectively, using a

UV-2401 spectrophotometer (Shimadzu Instruments, Inc., Columbia, MD, USA). The fractions with the high absorbance at 540 nm were pooled and referred to as 'Mb'. To locate Hb and Mb peaks, Hb from bighead carp blood was loaded onto a Sephadex G-100 column and the retention time was used to differentiate between Hb and Mb.

8.3.3.2 Preparation of Hb

Hb was prepared as per the method of Fyhn *et al.* (1973). Four volumes of cold 1.7% NaCl in 1 mM Tris buffer (pH 8.0) were added to heparinized blood. The mixture was centrifuged at 700*g* for 10 min at 4ºC. After the plasma was removed, the red blood cells were washed by suspending three times in 10 volumes of the same buffer. Red blood cells were lyzed in 3 volumes of 1 mM Tris (pH 8.0) for 1 h. One-tenth volume of 1 M NaCl was then added to aid in stromal removal before ultracentrifugation at 28,000*g* for 15 min at 4ºC. Low molecular mass components (<10 kDa) were removed from 'Hb' using a prepacked PD-10 column (column volume: 13.5 ml, medium volume: 8.3 ml) (Bio-Rad, Hercules, CA, USA) and 50 mM Tris buffer (pH 8.5) was used as an elution buffer with the gravity flow.

8.3.3.3 Preparation and quantification of oxyMb and oxyHb

OxyMb and oxyHb were prepared according to the method of Tang *et al.* (2004) with some modifications. An aliquot (3 ml; 0.1 mM heme) of Mb or Hb solutions was added with 1.5 mg of sodium dithionite. After stirring for 5 min, sodium dithionite was removed by a prepacked PD-10 column and 20 mM phosphate buffer (pH 7.0) was used for elution with the gravity flow. The concentrations of oxyMb and oxyHb solutions were determined by measuring the absorbance at 525 nm. The molar extinction coefficient of 7.6×10^{-3} was used for the calculation (Tang *et al.*, 2004) and the concentration was expressed as μM heme protein.

8.3.4 Heme release from bighead carp Mb and Hb during storage

8.3.4.1 Preparation of apo Streptococcal heme-associated protein (apoShp)

Shp^{229} plasmid was supplied by Dr. Benfang Lei (Montana State University, Bozeman, MT, USA). Cell transformation and expression were performed as described by Aranda IV *et al.* (2007) and Zhu, Liu and Lei (2008). Shp^{229} was transformed into *Escherichia coli* BL21 (DE3) and added with SOC medium (Novagen, Madison, WI, USA). Bacteria were cultured in Luria-Bertani broth supplemented with 100 mg/l ampicillin at 37ºC. Transformed

bacteria were combined with freezing medium and then kept as glycerol stocks of transformed bacteria at -80ºC until use. For expression, transformed bacteria were cultured in 12 l of Terrific broth supplemented with 100 mg/l ampicillin at 37ºC and Shp expression was induced with 0.5 mM IPTG for 4 h. Bacteria were harvested by centrifugation at 3,125 rpm for 15 min. The cell pellet was resuspended in 50 ml of 20 mM Tris-HCl buffer (pH 8.0). Portion of apoShp was in inclusion bodies and recovered as pellet after lysis by sonication (50% Duty cycle, 40% Power). Insoluble Shp was dissolved in 50 ml of 8 M urea and the denatured Shp was refolded by dialysis against 4 l of 20 mM Tris-HCl buffer (pH 8.0) with 3 changes at 4ºC.

The refolded apoShp was loaded onto a DEAE column (2 × 8 cm). The column was washed with 50 ml of 20 mM Tris-HCl buffer (pH 8.0) and eluted with 500 ml-linear gradient of 0-0.25 M NaCl. The fractions containing apoShp were subjected to ammonium sulfate fractionation (0-70% saturation). The precipitate obtained after centrifugation at 20,000*g* for 10 min was dissolved in a minimal volume of 20 mM Tris-HCl buffer (pH 8.0) and then dialyzed against 20 volumes of the same buffer. The sample was loaded onto a SP-Sepharose column (2 × 6 cm). The column was washed with 50 ml of 20 mM Tris-HCl buffer (pH 8.0) and eluted with 100 ml-linear gradient of 0.04-0.2 M NaCl. The fractions containing purified apoShp were pooled, dialyzed against 20 mM Tris-HCl buffer (pH 8.0) and then concentrated using Amicon Ultra-15 Centrifugal Filter Units, 30 kDa (Millipore, Billerica, MA, USA). During purification, apoShp was identified by measuring the absorbance at 280 nm and determining protein patterns using SDS-PAGE (Laemmli, 1970; Zhu *et al.*, 2008). To determine the concentration of apoShp, the absorbance at 280 nm and molar extinction coefficient of 28×10^{-3} were used for calculation.

8.3.4.2 Determination of heme release

Heme released from metMb and metHb to apoShp were monitored, following the method of Zhu *et al.* (2008) and Nygaard *et al.* (2006) with some modifications. To prepare metMb and metHb, an aliquot (3 ml; 0.1 mM heme) of Mb or Hb solutions was added with 1.5 mg of potassium ferricyanide (Tang *et al.*, 2004). Then, potassium ferricyanide was removed using a prepacked PD-10 column. MetMb or metHb (3.5 μM) was reacted with apoShp (7 μM) in 50 mM phosphate buffer, pH 6.0 at 4ºC. During 9 days of refrigerated storage, the mixtures were scanned from 380 to 700 nm, in which peaks at 420, 530 and 570 nm indicated the transfers of

met-heme into apoShp. For the controls, metMb and metHb without the addition of apoShp were performed under the same condition.

8.3.5 Effect of different phenolic compounds on lipid oxidation mediated by Mb and Hb in washed mince

8.3.5.1 Preparation of washed mince

Washed mince from bighead carp was prepared following the method of Grunwald *et al.* (2006) with a slight modification. The mince was washed twice with cold distilled water with a mince/water ratio of 1:3 (w/v). The mixture was stirred continuously for 2 min with a glass rod and allowed to settle for 15 min. The mixture was filtered using 2 layers of cheese-cloth. Thereafter, 3 volumes of cold 50 mM sodium phosphate buffer (pH 6.0) were mixed with washed mince, stirred and filtered as described previously twice. The retentate was mixed with a designated buffer with a mince/buffer ratio of 1:1 (w/v) and homogenized for 3 min at a speed of 13,000 rpm. The pH of muscle slurry was checked and adjusted to pH 6.0$\pm$0.05 using 0.5 M NaOH or 0.5 M HCl. After standing for 15 min, the mixture was centrifuged at 15,000*g* for 25 min. The pellet was collected and referred to as 'washed mince'. All processes were performed at 4ºC. Washed mince was packaged in polyethylene bag, vacuum-sealed and stored at -80ºC. The sample was stored not longer than 3 weeks.

8.3.5.2 Preparation of washed mince containing Mb/Hb and different phenolic compounds

Washed mince was thawed overnight at 4ºC and ground until the homogeneity was obtained using a Hamilton Beach Custom Grind Type CM04 coffee grinder (Hamilton Beach/Proctor Silex, Inc., Southern Pines, NC, USA). The sample (20 g) was transferred to an amber vial and added with 50 mM phosphate buffer (pH 6.0) to obtain the final moisture content of 90%. In order to inhibit the microbial growth, streptomycin sulfate with a final concentration of 200 ppm was added to the washed mince.

Different phenolic compounds including caffeic acid, gallic acid and tannic acid (20 mg) were added to distilled water (10 ml). The mixtures were adjusted to pH 6.0 using 2 M NaOH. The solutions were separately added into washed mince to obtain a final concentration of 200 ppm. This concentration showed the highest *in vitro* antioxidative properties including ABTS

radical scavenging activity, ferric reducing antioxidant power and metal chelating activity without the interfering effect on color of mixture (data not shown). OxyMb or oxyHb solution was then added into washed mince to obtain a final concentration of 24 μmol heme/kg mince. For the control sample, phosphate buffer was added instead of phenolic compounds and/or heme protein solution. The mixtures in vial were mixed thoroughly with a plastic spatula. All samples were stored on ice for 9 days. Molten ice was removed and the same amount of ice was replaced every day. Samples were taken at day 0, 1, 3, 5, 7 and 9 of storage for analyzes, except for hexanal analysis, where the samples were taken at day 0, 5 and 9.

8.3.5.3 Analyzes

8.3.5.3.1 Measurement of redness

Colorimetric values of washed mince with different treatments were determined using a Minolta CR-300 Chroma Meter (Minolta Camera Co., Osaka, Japan). The aperture size was 1 cm. Illuminant C was used. A white calibration plate was used to calibrate the instrument. Washed mince samples were determined for *a*-value (redness/greenness).

8.3.5.3.2 Measurement of peroxide value (PV)

PV was determined as described by Richards and Hultin (2000) with some modifications. Washed mince (1 g) was mixed with 10 ml of a chloroform/methanol mixture (1:1, v/v). The mixture was homogenized at 13,500 rpm for 2 min and then added with 3.08 ml of 0.5% NaCl. The mixture was vortexed at a moderate speed for 30 s and centrifuged at 1,800*g* for 6 min. Using a glass syringe, a 2 ml of the lower phase was transferred to a screw-cap tube and then added with 1.33 ml of cold chloroform/methanol (1:1) mixture, 25 μl of 30% (w/v) ammonium thiocyanate and 25 μl of 20 mM ferrous chloride in 3.5% HCl. The reaction mixture was allowed to stand for 20 min at room temperature and the absorbance at 500 nm was read. A standard curve was prepared using cumene hydroperoxide at concentrations ranging from 0 to 20 μM. PV was calculated and expressed as μmol cumene hydroperoxide/kg sample.

8.3.5.3.3 Measurement of thiobarbituric acid reactive substances (TBARS)

TBARS were determined following the method of Buege and Aust (1978). The sample (0.5 g) was homogenized with 2.5 ml of a solution containing 0.375% thiobarbituric acid (w/v), 15% trichloroacetic acid (w/v) and 0.25 M HCl. The mixture was heated at 105ºC for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3,600*g* at 25ºC 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 40 μM. TBARS values were calculated and expressed as μmol malonaldehyde/kg sample.

8.3.5.3.4 Measurement of hexanal content

Hexanal content was determined using a solid-phase micro-extraction (SPME) technique and gas chromatography (GC). The 10 ml-vial (Agilent Technologies, Palo Alto, CA, USA) with a metal cap and hole PTFE/silicone septa (MicroLiter Analytical Supplies Inc., Suwanee, GA, USA) containing 1 g of frozen sample was allowed to stand at room temperature for 15 min, followed by heating at 40ºC for 5 min. The SPME fiber (PDMS/DVB, fused silica, d_f 65 μm, needle size 23 ga) (Supelco, Bellefonte, PA, USA) was conditioned at 260ºC for 30 min before use and then exposed to headspace of vial at 40ºC for 10 min.

GC analysis was performed using a HP 6890 gas chromatography (Hewlett-Packard, Palo Alto, CA, USA) equipped with capillary column (DB-5, 30 m length × 0.25 mm i.d. × 0.1 μm film thickness) and flame ionization detector (FID). After injection of SPME fiber into GC/FID injection port, hexanal from samples was desorbed from SPME fiber at 250ºC for 5 min. The flow rate of carrier gas was 1 ml/min. Inlet and detector temperatures were 250 and 270ºC, respectively. The oven temperature was programmed at 40ºC for 5 min with a 10ºC/min ramp rate until 90ºC. Identification of hexanal was based on the retention time. Quantification of hexanal in the sample was made using a standard curve of hexanal solution (0-0.25 ppm).

8.3.6 Statistical analysis

Data from triplicate run were subjected to analysis of variance (ANOVA) with the confidence level of 95%. Comparison of means was carried out by Duncan's multiple range test (Steel and Torrie, 1980). For pair comparison, T-test was used. Statistical analysis was performed using the Statistical Package for Social Science (SPSS 14.0 for windows, SPSS Inc., Chicago, IL, USA).

8.4 Results and discussion

8.4.1 Heme release from bighead carp Mb and Hb during storage

Changes in absorption spectra (380-450 nm and 450-700 nm) of metMb and metHb treated with apoShp and stored for different times are shown in Figure 39. ApoShp, a protein without chromophore, could acquire ferric heme released from heme protein and the oxidized Shp exhibited the typical spectrum (Zhu *et al.*, 2008). Oxidation state of Shp had Soret peak at 420 nm and two additional peaks at 530 and 570 nm (Nygaard *et al.*, 2006). For metMb treated with apoShp, no changes in Soret peak (406 nm) as well as typical spectra of metMb in a region of 450-700 nm were observed throughout the storage. Conversely, changes in absorption spectra for both regions of metHb treated with apoShp were obtained during 9 days of storage. Soret peak of treated metHb shifted from 405 nm to 421 nm with increasing storage time up to 3 days and was likely stable until the end of storage. In a region of 450-700 nm, a major peak at 529 nm and a minor peak at 560 nm were observed as storage time increased. These changes are likely due to heme transfer from metHb into apoShp. The present study revealed that heme was strongly stabilized within metMb structure during storage. Conversely, metHb was not stable, as indicated by a rapid heme release with increasing storage. Richards *et al.* (2005) demonstrated that anodic Hb, which had rapid heme loss rate, was a better catalyst of lipid oxidation in washed cod muscle, compared to Mb. Thus, it can be expected that Hb from bighead carp should promote lipid oxidation more effectively than carp Mb due to relatively rapid heme release from Hb.

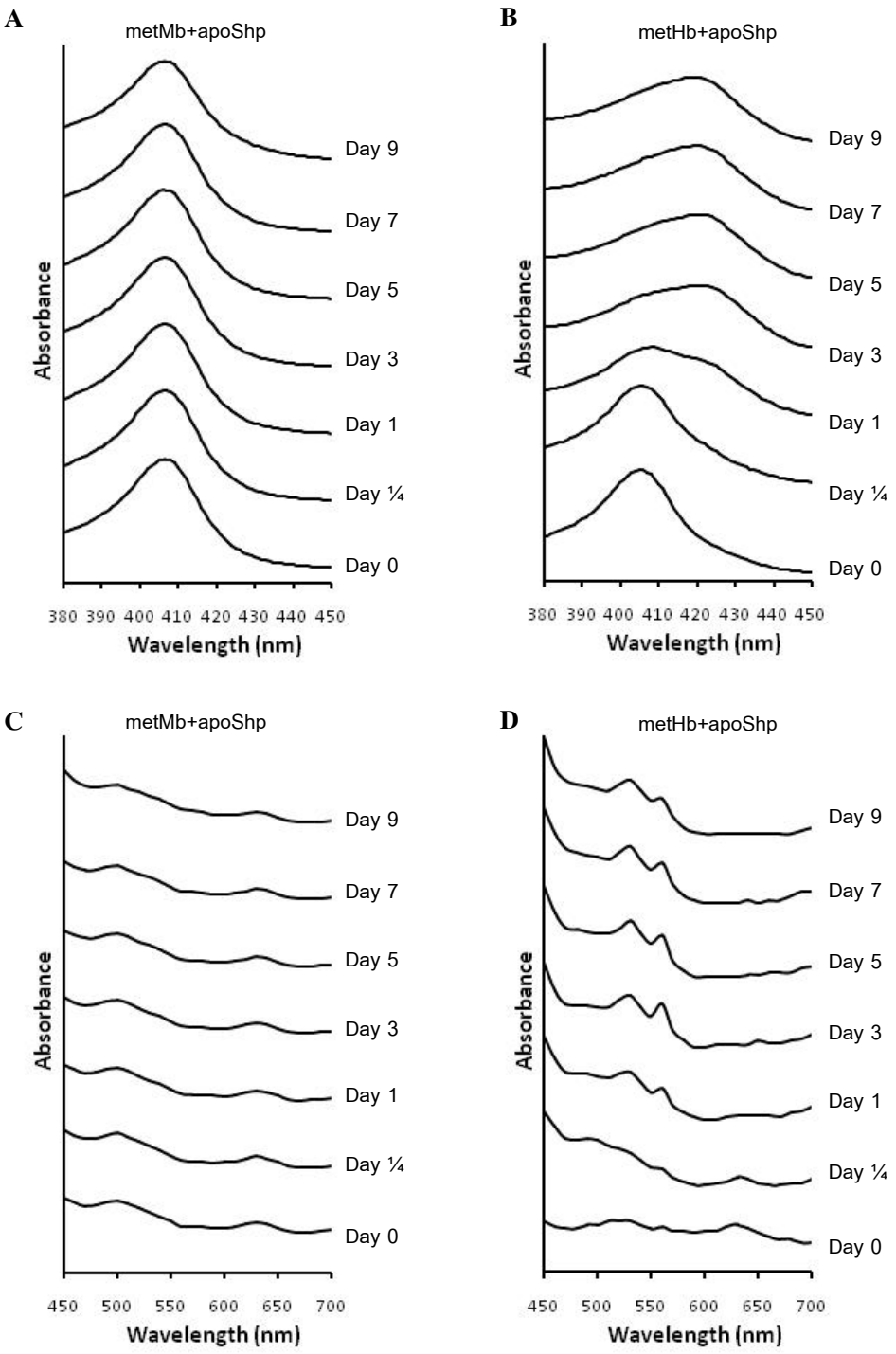


Figure 39. Changes in absorption spectra in a region of 380-450 nm (A and B) and 450-700 nm (C and D) of metMb and metHb treated with apoShp, pH 6.0 during 9 days of refrigerated storage

8.4.2 Effect of different phenolic compounds on lipid oxidation in washed mince mediated by Mb and Hb from bighead carp

8.4.2.1 Changes in redness

Redness (*a*-value) of washed mince (C), those containing Mb without (M) and with different phenolic compounds including caffeic acid (MC), gallic acid (MG), tannic acid (MT) and those containing Hb without (H) and with caffeic acid (HC), gallic acid (HG) and tannic acid (HT) during 9 days of iced storage are shown in Figure 40. The C sample had the low *a*-value and it remained unchanged throughout 9 days of storage ($P > 0.05$). All samples containing heme proteins showed the highest redness at day 0. With increasing storage time, the decreases in redness of all samples were observed up to the end of storage ($P < 0.05$), except for HC and HG samples, in which the constant *a*-values were observed after 1 day of storage. Mb and Hb are the major heme proteins found in fish, especially in the dark muscle and mostly display the red color when oxy-form is dominant (Baron and Andersen, 2002; Brown, 1962; Faustman and Cassens, 1990). Decreases in redness were more likely due to the formation of metMb/Hb, resulting from the oxidation of oxyMb/Hb.

For samples containing Mb (Figure 40A), the redness of MT sample was more retained, compared with other samples. MC and MG samples had the lower redness than that containing only Mb during 7-9 days of storage ($P < 0.05$). The result suggested that tannic acid showed the highest efficiency in preventing oxidation of Mb during the extended storage. On the other hand caffeic acid and gallic acid might act as the pro-oxidant for the system containing Mb. Antioxidative activity of phenolic compounds varied upon their type and molecular structure (Medina *et al.*, 2007). Tannic acid might reduce ferric (Fe^{3+}) into ferrous (Fe^{2+}), thereby lowering Mb oxidation. Additionally, the discoloration of washed mince at extended storage might be due to the oxidation of caffeic acid and gallic acid. Some oxidized phenolic compounds or quinone could be generated during preparation, processing and storage (Prigent *et al.*, 2007). Quinone has been reported for both antioxidative and pro-oxidative activities (He *et al.*, 2003). Therefore, differences in molecular structure of phenolic compounds might be associated with oxidation state of Mb, which determined the color of washed mince.

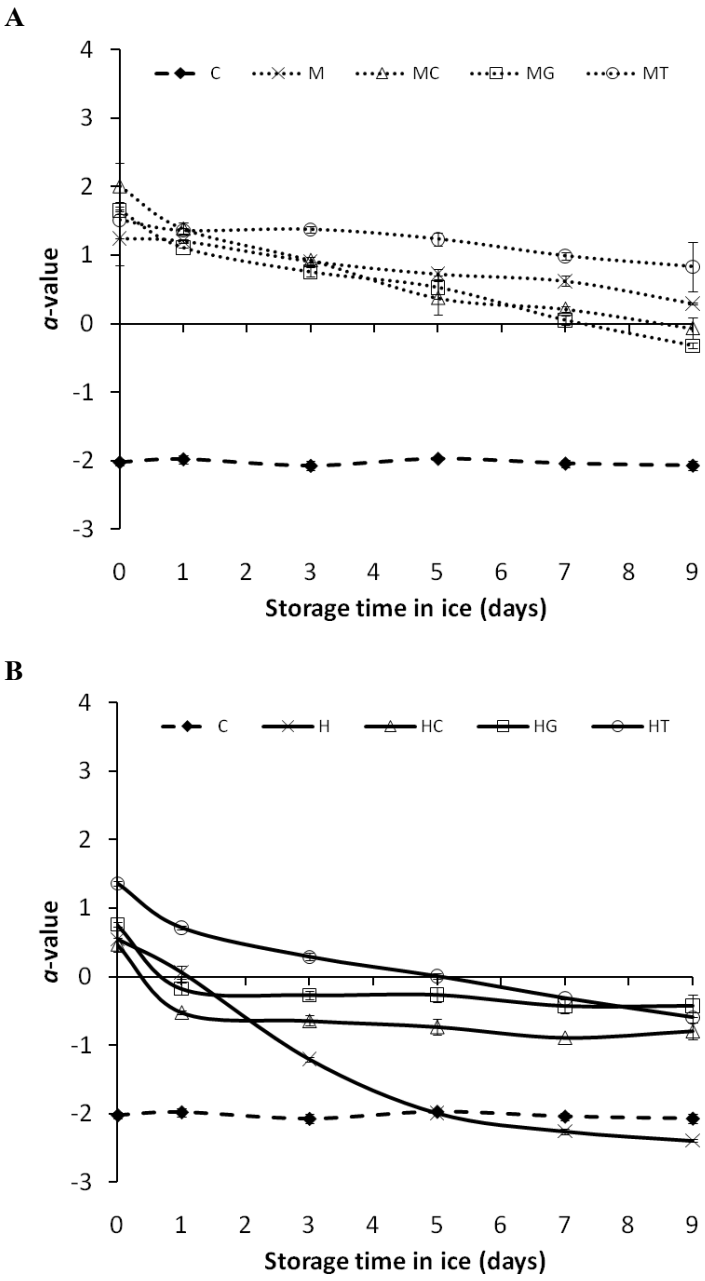


Figure 40. Changes in redness (*a*-value) of washed mince containing Mb (A) and Hb (B) without and with caffeic acid, gallic acid or tannic acid at a level of 200 ppm during 9 days of iced storage, pH 6.0. Bars represent standard deviation (n=3). C : washed mince, M : washed mince containing Mb, MC : M+caffeic acid, MG : M+gallic acid, MT : M+tannic acid, H : washed mince containing Hb, HC : H+caffeic acid, HG : H+gallic acid, HT : H+tannic acid

When Hb was incorporated into the washed mince, the highest redness was observed in HT sample within the first 5 days, compared with all samples containing Hb ($P < 0.05$) (Figure 40B). During 7-9 days of storage, no differences in redness between HT and HG samples were found ($P > 0.05$). The result suggested that tannic acid exhibited the most efficacy in retardation of Hb oxidation in the early stage of storage (the first 5 days). H sample had the continuously decrease in redness throughout the storage time of 9 days ($P < 0.05$). However, HC and HG samples had the negligible changes in redness after 1 day of storage ($P < 0.05$). The result indicated the susceptibility of Hb to autoxidation with increasing storage time. Although caffeic acid and gallic acid could not prevent the oxidation of Hb in the early stage of storage, they could maintain the redness of washed mince at the later storage. Therefore, tannic acid was considered as the most potent compound, compared with caffeic acid and gallic acid in retardation of Mb and Hb oxidation as well as stabilization of color of washed mince.

8.4.2.2 Changes in PV

PV of washed mince without and with the addition of Mb/Hb and different phenolic compounds during 9 days of iced storage are shown in Figure 41. For washed mince (C sample), slight increases in PV were observed with increasing storage time ($P < 0.05$). Washing process could remove some pro-oxidants as well as some lipids from the muscle (Grunwald and Richards, 2006), leading to the lowered lipid oxidation occurring in washed mince. Unsaturated fatty acids in fish muscle are oxidized easily during storage (Thiansilakul *et al.*, 2010). Marked increases in PV were found in M sample with increasing storage time up to 5 days, followed by a decrease thereafter ($P < 0.05$) (Figure 41A). Slight increases in PV were observed for samples added with Mb in the presence of phenolic compounds (MC, MG and MT) with increasing storage time. At day 5, MC sample had the lowest PV, compared with others ($P < 0.05$). Thus, Mb exhibited a strong pro-oxidative activity in washed mince during storage. The addition of phenolic compounds was able to retard the formation of hydroperoxides in washed mince containing Mb, but the effectiveness varied with types of phenolic compounds.

Washed mince added with Hb (H sample) had the marked increases in PV up to 5 days of storage and slight decreases in PV were obtained thereafter ($P < 0.05$) (Figure 41B). Higher PV was found in H sample throughout the storage, compared with other samples ($P < 0.05$). It was noted that Hb exhibited a greater pro-oxidative effect on lipid oxidation in washed

mince than did Mb ($P < 0.05$). This was probably due to a greater release of heme in Hb than Mb (Figure 39). According to a sharp decrease in redness of H sample during storage, the oxidized form, metHb, was plausibly related with the accelerated lipid oxidation. In the presence of phenolic compounds, the formation of PV in washed mince induced by Hb was suppressed. For HT sample, the increase in PV was noted after 3 days of storage, followed by a slight decrease at day 7 ($P < 0.05$). The lowest PV were found in HC and HG samples during storage, compared with others ($P < 0.05$).

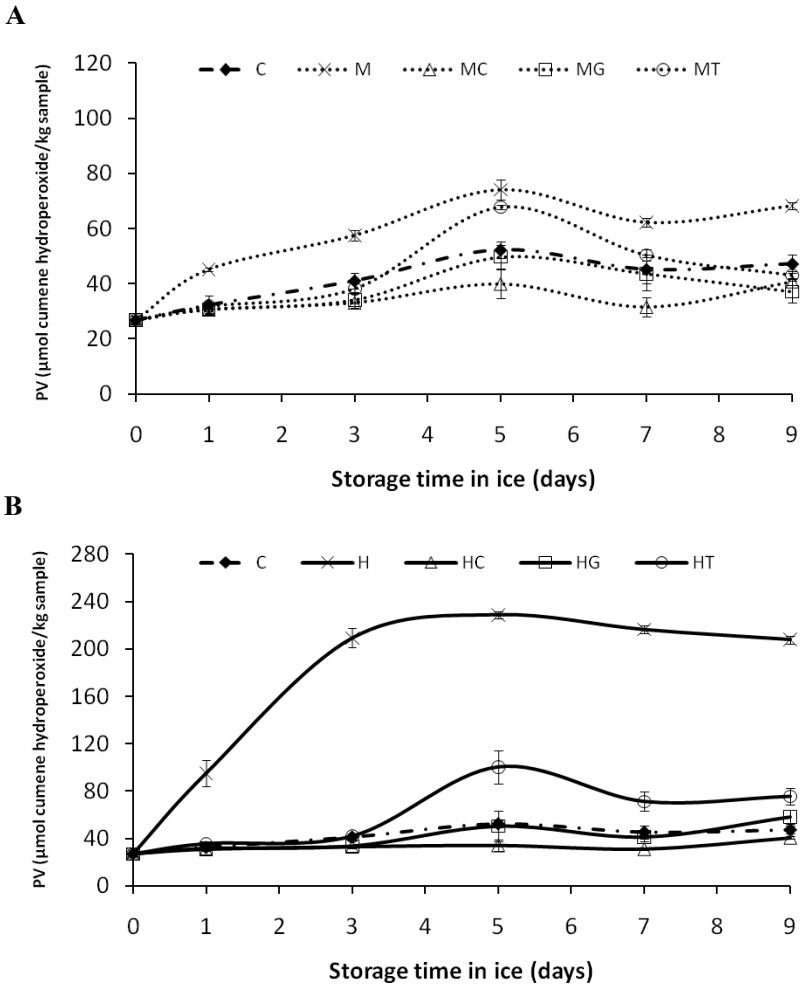


Figure 41. Changes in PV of washed mince containing Mb (A) and Hb (B) without and with caffeic acid, gallic acid or tannic acid at a level of 200 ppm during 9 days of iced storage, pH 6.0. Bars represent standard deviation (n=3). Key: see Figure 40 caption

The oxidation of oxy- to met-heme protein generates the reactive intermediates, which were capable of enhancing further oxidation of unsaturated fatty acids (Faustman *et al.*, 2010). Hydroperoxides are primary products generated during lipid oxidation. Decreases in PV at the advancement of storage indicated the decomposition of hydroperoxides to other compounds (Nawar, 1996; Thiansilakul *et al.*, 2011a). Although tannic acid was able to maintain redness of samples more effectively than caffeic acid and gallic acid, it had the lower ability in retardation of lipid oxidation. After donation of electron to met-heme protein, tannic acid might have less reducing power, thereby lowering its antioxidative activity. Phenolic compounds acted as antioxidant through different mechanisms, such as donating electron or hydrogen atom to radicals, scavenging of free radicals and chelation of transition metals, thus terminating the propagation of lipid oxidation. Caffeic acid, gallic acid and tannic acid with a concentration of 200 ppm had a great ability to chelate iron (data not shown), which might be released from heme protein during storage (Thiansilakul *et al.*, 2010; Thiansilakul *et al.*, 2011b).

8.4.2.3 Changes in TBARS

TBARS of washed mince without and with the addition of Mb/Hb and different phenolic compounds during 9 days of iced storage are displayed in Figure 42. TBARS value has been used to determine the lipid oxidation secondary products, which were decomposed from hydroperoxides. For M sample, TBARS increased as storage time increased, especially during 3-5 days of storage ($P < 0.05$) (Figure 42A). MC, MG and MT samples had negligible changes in TBARS within the first 3 days of storage and the gradual increases in TBARS were obtained thereafter ($P < 0.05$). During storage, similar TBARS values were observed among MC, MG and MT samples, except for day 9, in which a higher TBARS value was found in MT sample ($P < 0.05$). Higher TBARS values were observed in M sample, compared with other samples containing Mb throughout storage ($P < 0.05$). Therefore, addition of phenolic compounds into washed mince could minimize the formation of TBARS induced by Mb during storage.

For H sample, the marked increases in TBARS were noticed during 1-5 days of storage and slight increases in TBARS were obtained thereafter ($P < 0.05$) (Figure 42B). Hb effectively accelerated lipid oxidation in washed mince as indicated by the highest TBARS of H sample throughout the storage, compared with other samples containing Hb. For HC, HG and HT samples, no changes in TBARS were noticeable within the first 3 days of storage ($P > 0.05$).

Thereafter, gradual increases in TBARS were observed until the end of storage ($P < 0.05$). HC and HG samples generally had the lower TBARS than did HT sample, suggesting that both caffeic acid and gallic acid showed higher antioxidative activity than tannic acid.

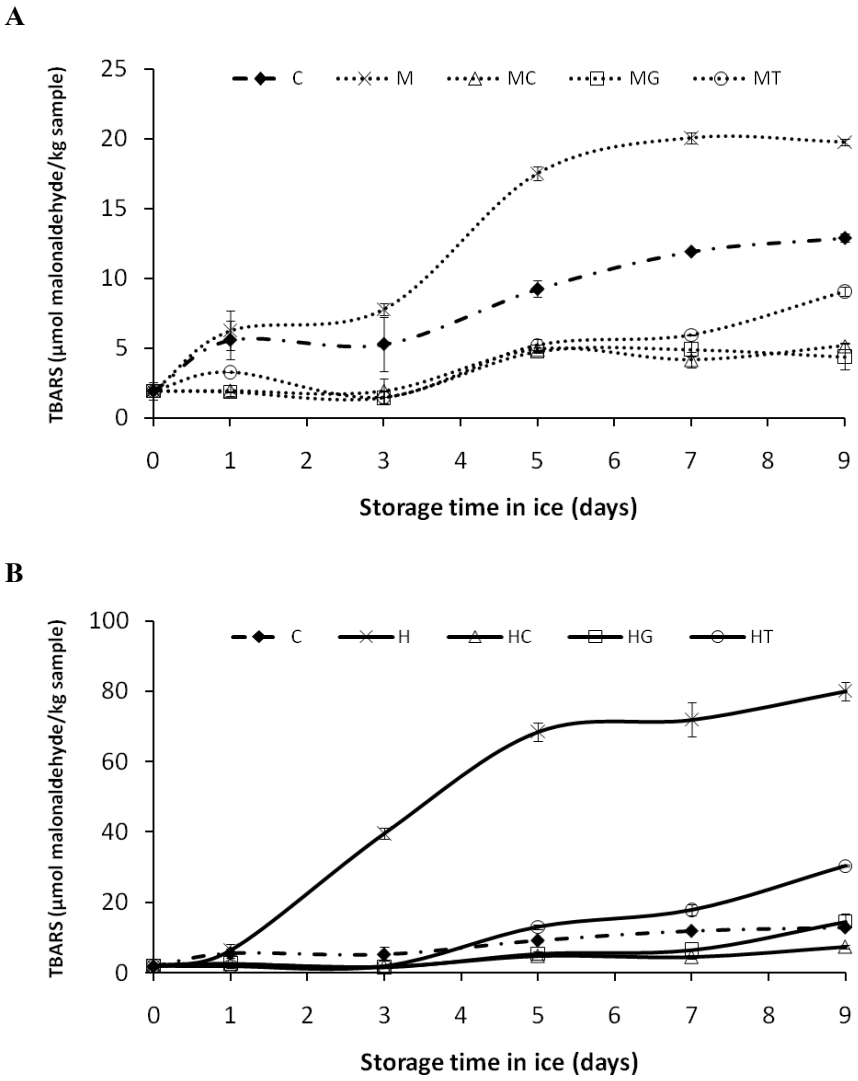


Figure 42. Changes in TBARS of washed mince containing Mb (A) and Hb (B) without and with caffeic acid, gallic acid or tannic acid at a level of 200 ppm during 9 days of iced storage, pH 6.0. Bars represent standard deviation (n=3). Key: see Figure 40 caption

Based on TBARS values, different phenolic compounds were able to prevent lipid oxidation process almost completely within the first 3 days of storage. Thereafter, caffeic acid and gallic acid likely exhibited the better antioxidative activity than did tannic acid for both Hb- and Mb-mediated lipid oxidations. However, the greater decreases in redness of samples containing Mb/Hb added with caffeic acid and gallic acid were observed, compared with those added with tannic acid during storage. It indicated that the formers had less preventive effect on Mb/Hb oxidation than lipid oxidation. Molecular weight of caffeic acid and gallic acid are about 10-time lower than of tannic acid and possibly distributed into washed mince more uniformly. As a result, it could act as antioxidant toward lipids more effectively. Antioxidative properties of phenolic compounds varied, depending on many factors such as the intrinsic property, the molecular size, the location and orientation in targeted substrate as well as the interactions with other food ingredients (Decker *et al.*, 2005; Kathirvel and Richards, 2009; Lee *et al.*, 2006; Wang *et al.*, 2010). Medina *et al.* (2007) reported that the prevention of lipid oxidation in fish muscle by hydroxyl cinnamic acids (i.e. caffeic acid, chrologenic acid) was more governed by the capacity of donating electrons than the ability for chelating metals or the polarity.

8.4.2.4 Changes in hexanal content

Hexanal contents of washed mince without and with the addition of Mb/Hb and different phenolic compounds during 9 days of iced storage are shown in Figure 43. Hexanal contents in all samples increased after 5 days of storage, but the subsequent decrease was found at day 9 of storage ($P < 0.05$). Both saturated and unsaturated fatty acids were found in the ordinary muscle of bighead carp, in which palmitic acid (C16:0) was the most abundant fatty acid, followed by oleic acid (C18:1(*n*-9)) and docosahexaenoic acid (C22:6(*n*-3)) (data not shown). Unsaturated fatty acids in fish muscle are susceptible to oxidation during the extended storage, leading to a formation of several derivatives such as aldehyde, ketone and alcohol (Thiansilakul *et al.*, 2010; Thiansilakul *et al.*, 2011a). Hexanal was a major product found in washed seabass without and with the addition of Mb during storage. However, the sample containing Mb had the higher hexanal content. Hexanal formed was related with fishy and rancid odor development (Thiansilakul *et al.*, 2011a).

During storage, the higher hexanal contents were found in M sample, compared with C sample ($P < 0.05$) (Figure 43A). Different phenolic compounds were able to lower hexanal

contents in washed mince containing Mb. At the end of storage, caffeic acid showed higher preventive effect on hexanal formation induced by Mb, followed by gallic acid and tannic acid, respectively ($P < 0.05$).

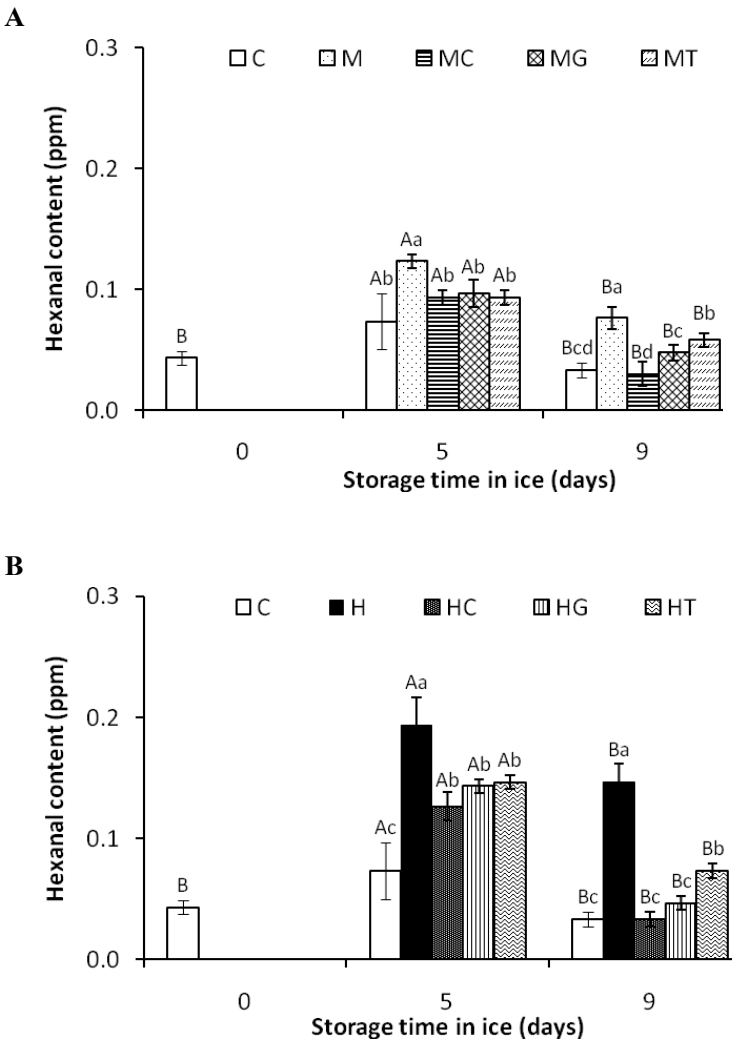


Figure 43. Changes in hexanal content of washed mince containing Mb (A) and Hb (B) without and with caffeic acid, gallic acid or tannic acid at a level of 200 ppm during 9 days of iced storage, pH 6.0. Different capital letters within the same sample indicate significant differences. Different letters within the same day indicate significant differences. Bars represent standard deviation (n=3). Key: see Figure 40 caption

For washed mince containing Hb (Figure 43B), higher hexanal content was found in sample added with Hb, compared with that added with Mb (Figure 43A). Higher release of heme from Hb might lead to the higher pro-oxidative activity, compared with Mb (Figure 39). The addition of phenolic compounds also exhibited the inhibitory effect on hexanal formation in washed mince. Caffeic acid and gallic acid showed a better prevention than did tannic acid at day 9 of storage ($P < 0.05$). Different phenolic compounds, especially caffeic acid and gallic acid, effectively prevented lipid oxidation in fish muscle during storage.

8.5 Conclusions

During 9 days of iced storage, washed bighead carp containing Mb and Hb underwent discoloration and lipid oxidation to a great extent. A stronger pro-oxidative activity of Hb, compared with Mb, was likely due to a rapid autoxidation and higher heme release from Hb. In the presence of Mb and Hb, the addition of tannic acid could lower discoloration of washed mince during storage, whereas caffeic acid and gallic acid were not able to maintain redness of washed mince. Lipid oxidation as well as off-odor development mediated by Mb and Hb were lowered when different phenolic compounds were incorporated. Caffeic acid and gallic acid generally served as the better antioxidants than did tannic acid. Therefore, selected phenolic compounds should be promising to retard lipid oxidation and discoloration in fish muscle during storage.

CHAPTER 9

Effect of phenolic compounds in combination with modified atmospheric packaging on retardation of quality losses of refrigerated Eastern little tuna slices

9.1 Abstract

Color, lipid oxidation, microbial load and sensory property of Eastern little tuna (*Euthynnus affinis*) slices treated without and with caffeic acid or tannic acid (200 ppm) during 15 days of storage at 4ºC in air or under modified atmospheric packaging (MAP:60%CO_2, 35%N_2 and 5%O_2) were studied. Lipid oxidation as well as undesirable color and odor of tuna slices stored in air (A) increased as the storage time increased ($P < 0.05$). Tannic acid exhibited a greater preventive effect on metMb formation and lipid oxidation than did caffeic acid ($P < 0.05$). Samples treated with tannic acid and kept under MAP (MT) had the lowest peroxide value, thiobarbituric acid-reactive substances and fishy odor intensity, with coincidentally higher odor, color and overall likeness score than others ($P < 0.05$). After 12 days of storage, changes in unsaturated fatty acids, especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), were lower in MT, compared with A sample. Based on microbiological acceptability limit (10^7 cfu/g), the shelf-life of tuna kept in air and MAP was estimated to be 6 and 12 days, respectively, irrespective of caffeic acid or tannic acid treatment. Therefore, tannic acid exhibited a synergistic effect with MAP on retardation of metMb formation, lipid oxidation and microbial growth, thereby improving the acceptance and increasing the shelf-life of tuna slices during refrigerated storage.

9.2 Introduction

Odor and color of fish are the important quality attributes for purchasing decision of consumers. High content of polyunsaturated fatty acids (PUFA) in fish flesh is associated with the development of undesirable odors and poor qualities due to the susceptibility to lipid oxidation (Chaijan *et al.*, 2006; Thiansilakul *et al.*, 2010). Myoglobin (Mb), a major heme protein in dark muscle contributing to the apparent color of meat, has a close relationship with

lipid oxidation (Thiansilakul *et al.*, 2011a). Pivarnik *et al.* (2011) reported that loss of redness and lipid oxidation in tuna steaks increased during room temperature, refrigerated and iced storage.

To obtain the fish with high quality during storage, antioxidants have been used to retard lipid oxidation and off-odor development. Phenolic compounds, the secondary metabolites present typically in plants, have been reported for their antioxidative activity by donating electrons and chelating metals (Medina *et al.*, 2007). Different structures and number of hydroxyl groups of phenolic compounds bring about the varying antioxidative property. Tang *et al.* (2001) reported the higher antioxidant effect of tea catechin in meat, poultry and fish than α-tocopherol at the same concentration (300 ppm) used. Corresponding to the ability of electron donating, caffeic acid showed the most preventive effect on lipid oxidation in minced horse mackerel, compared with other hydroxycinnamic acids and catechins (Medina *et al.*, 2007).

Modified atmospheric packaging (MAP) has been proved to be an effective preservation method, in which the shelf-life extension and quality retention of fish and fish products can be achieved (Masniyom *et al.*, 2002; Tang *et al.*, 2001). MAP with a high level of oxygen (70-80%) is used to preserve the bright red color of meat. However, oxygen at high level induced the oxidation of both lipid and Mb (Kim *et al.*, 2010). Thiansilakul *et al.* (2011b) found that high concentration of oxygen likely converted the purified oxyMb to metMb and induced a higher discoloration of Eastern little tuna fillet, compared with vacuum condition. MAP under a high level of CO_2 (40-100%) is used to inhibit bacterial growth and extend shelf-life of fish and fishery products (Sivertsvik *et al.*, 2002). Using MAP (60%CO_2, 35%N_2 and 5%O_2) in combination with tannic acid treatment (200 ppm) lowered lipid oxidation, heme protein changes and microbial growth as well as improved the sensory acceptance of refrigerated striped catfish slices (Maqsood and Benjakul, 2010).

Eastern little tuna (*Euthynnus affinis*) is a species available in the Gulf of Thailand and the Indian Ocean with the volume of 22,220 metric tons and a value of 18 million US dollars in 2008 which provide the high global economic value for canning and sashimi (Fisheries Foreign Affairs Division, 2008). To prolong the shelf-life of fish with minimized quality losses during storage, suppression of lipid and Mb changes is required. The objective of this investigation was to study the effect of phenolic compounds, caffeic acid or tannic acid, on

quality changes of Eastern little tuna slices kept under different packaging atmospheres during refrigerated storage.

9.3 Materials and methods

9.3.1 Chemicals

2-thiobarbituric acid and 1,1,3,3-tetramethoxypropane were purchased from Sigma (St. Louis, MO, USA). Cumene hydroperoxide was procured from Fluka (Buchs, Switzerland). Ammonium thiocyanate and ferrous chloride were obtained from Riedel (Seeize, Germany). Sodium chloride, trichloroacetic acid, chloroform and methanol were procured from Merck (Damstadt, Germany). Plate count agar (PCA) and Man Rogosa Sharpe (MRS) broth were obtained from Hi-media (Mumbia, India). All chemicals used were of analytical grade.

9.3.2 Fish samples

Eastern little tuna (*E. affinis*) with an average weight of 0.4-0.45 kg off-loaded after 24 to 36 h of capture were obtained from the dock in Songkhla province, Thailand. The fish were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within 1 h. Upon arrival, fish were washed with cold tap water, filleted, de-skinned and cut into slices with a weight of 50 g (thickness ~1.5-2 cm). The fish were placed on ice until use but not longer than 2 h.

9.3.3 Effect of caffeic acid or tannic acid in combination with MAP on quality changes of Eastern little tuna slices during refrigerated storage

Caffeic acid and tannic acid were dissolved separately in distilled water to gain a concentration of 10 mg/ml. The mixtures were adjusted to pH 7.0 using 2 M NaOH. One ml of caffeic acid or tannic acid (10 mg/ml) was applied uniformly to Eastern little tuna slices (50 g) to obtain a final concentration of 200 ppm. Fish slices were placed on the 20 × 12 cm^2-polystyrene trays (2 slices per tray) and inserted in 17.78 × 27.94 cm^2-nylon/polyethylene bag with the selected gas permeability (O_2 transmission rate of 0.66 g m^{-2} day^{-1} at 23ºC, 101.33 kPa). The samples were packaged under MAP (60%CO_2/5%O_2/35%N_2) with a gas/sample ratio of 2:1 (v/w)

using a Henko-vac type 1000 machine (Technovac, Italy). Fish slices treated without and with caffeic acid or tannic acid were packaged in air and designated as A, AC and AT, respectively, while those packaged under MAP were referred to M, MC and MT, respectively For the control samples (A and M), distilled water (1 ml) was added to the slices. All samples were stored at 4ºC and taken for analyzes at day 0, 3, 6, 9, 12 and 15 days. The fish slices were ground to uniformity before analyzes, except for sensory analysis, in which the slices were used for analysis without grinding.

9.3.4 Determination of metMb formation

Mb in ground samples was extracted following the method of Benjakul and Bauer (2001). Ground sample (2 g) was added with 20 ml of cold 40 mM sodium phosphate buffer (pH 6.8). The mixture was homogenized at 13,500 rpm for 10 s using an IKA Labortechnik homogenizer (Selangor, Malaysia), followed by centrifugation at 3,000*g* for 30 min at 4ºC using an Allegra 25R centrifuge (Beckman Coulter, Palo Alto, CA, USA). The supernatant was filtered through Whatman No. 1 filter paper. The absorbances of Mb solutions were measured at 503, 525, 557 and 582 nm. MetMb formation was calculated following a modified Krzywicki's equation (Tang *et al.*, 2004) as follows:

$$[\text{metMb}] = -0.159R1 - 0.085R2 + 1.262R3 - 0.520$$

where $R_1 = A_{582}/A_{525}$, $R_2 = A_{557}/A_{525}$ and $R_3 = A_{503}/A_{525}$.

9.3.5 Measurement of redness

Colorimetric values of ground samples with different treatments were determined using a colorimeter (JP7100F, Juki Corp., Tokyo, Japan). The instrument was calibrated using a white and black standard. All samples were determined for a^*-value (redness/greenness).

9.3.6 Measurement of lipid peroxides

Peroxide value (PV) was determined as described by Richards and Hultin (2002) with some modifications. Ground sample (1 g) was mixed with 10 ml of a chloroform/methanol

mixture (2:1, v/v). The mixture was homogenized at 13,500 rpm for 2 min and then added with 3.08 ml of NaCl (0.5%). The mixture was vortexed at a moderate speed for 30 s and centrifuged at 1,800*g* for 6 min. Two ml of the lower phase was transferred to a screw-cap tube and then added with 1.33 ml of cold chloroform/methanol (2:1) mixture, 25 µl of 30% (w/v) ammonium thiocyanate and 25 µl of 20 mM ferrous chloride in 3.5% (w/v) HCl. The reaction mixture was allowed to stand for 20 min at room temperature and the absorbance at 500 nm was read. A standard curve was prepared using cumene hydroperoxide at concentrations ranging from 0 to 20 µM. PV was calculated and expressed as µmol cumene hydroperoxide/kg sample.

9.3.7 Measurement of thiobarbituric acid reactive substances (TBARS)

TBARS were determined following the method of Buege and Aust (1978). The sample (0.5 g) was homogenized with 2.5 ml of a solution containing 0.375% (w/v) thiobarbituric acid, 15% (w/v) trichloroacetic acid and 0.25 M HCl. The mixture was heated at 105ºC for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3,600*g* at 25ºC 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 40 µM. TBARS values were calculated and expressed as µmol malonaldehyde/kg sample.

9.3.8 Lipid extraction and determination of fatty acid profile

Lipids of fresh tuna, A and MT samples stored for 12 days were extracted following the method of Bligh and Dyer (1959) prior to analysis of fatty acid profile. The fatty acid methyl esters (FAMEs) were prepared according to the method of (AOAC, 2000). The prepared methyl ester was injected into the gas chromatography (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) at a split ratio of 10:1. A model of J&W 122-2362 DB-23 capillary column (50%-Cyanopropyl)-methylpolysiloxane, 60 m × 0.25 mm) (Chrom Tech, Inc., Apple Valley, MN, USA) was used. The analytical conditions were: injection port temperature of 250ºC and detector temperature of 270ºC. The oven was programmed from 170 to 220ºC at a rate of 1ºC/min. Retention times of FAME standards were used to identify chromatographic peaks of samples. Fatty acid content was calculated based on the peak area ratio and expressed as g fatty acid/100 g lipids.

9.3.9 Microbiological analysis

Fish slices with different treatments (25 g) were collected aseptically in a stomacher bag. After adding with 10 volumes of 0.85% (w/v) sterile saline solution, the samples were mixed using a Stomacher blender (Stomacher M400, Seward Ltd., Worthington, England) for 1 min and a series of 10-fold dilutions was made by the same diluent. Total viable count (TVC) and psychrophilic bacterial count (PBC) were determined by plate count agar (PCA) with the incubation at 35ºC for 2 days (Hasegawa, 1987) and 7ºC for 7 days (Cousin *et al.*, 1992), respectively. Lactic acid bacteria (LAB) count was also determined using MRS agar after 3 days of incubation at 37ºC (Ordonez *et al.*, 1991). Microbial counts were expressed as log cfu/g.

9.3.10 Determination of fishy odor and likeness

Fishy-odor intensity was evaluated by 6 trained panelists who had the extensive experience in the evaluation of off-odors of raw fish. Panelists were trained for 3 sessions with 1.5 h each by sniffing the different standards. The standards were prepared by keeping tuna slices at 4ºC for 0, 3, 6, 9 and 12 days, representing the standards for fishy-odor score of 0, 1, 2, 3 and 4, respectively. During evaluation, the samples were placed on plastic cups covered with aluminum foil and kept in ice. To test the samples, panelists were asked to open the aluminum foil, sniff the headspace above the samples and score the samples using a scale ranging from none (score=0) to strong (score=4) (Sohn *et al.*, 2005).

The evaluation of likeness was performed by 30 untrained panelists who were familiar with fish consumption. The assessment was conducted for the odor, color and overall likeness of fish slices using a 9-point hedonic scale: 1, dislike extremely; 2, dislike very much; 3, dislike moderately; 4, dislike slightly; 5, neither like nor dislike; 6, like slightly; 7, like moderately; 8, like very much; 9, like extremely.

9.3.11 Statistical analysis

Data from three different experiments were subjected to analysis of variance (ANOVA) with the confidence level of 95%. Comparison of means was carried out by Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 14.0 for windows, SPSS Inc., Chicago, IL, USA).

9.4 Results and Discussion

9.4.1 MetMb formation

Changes in %metMb of tuna slices treated without and with caffeic acid or tannic acid in combination without and with MAP are presented in Figure 44. At day 0, metMb in tuna slices were in a range of 31.4-34.9%. MetMb in all samples increased markedly within the first 6 days of storage ($P < 0.05$). For samples kept in air, negligible changes were obtained during day 6-9, but the decreases in %metMb occurred at day 12 of storage ($P < 0.05$). MC and MT samples had the gradual increases in %metMb after day 6 of storage, while no changes were obtained in M sample ($P > 0.05$). Nevertheless, slight decreases in %metMb were found in M and MC samples at day 15 ($P < 0.05$). Physiologically active forms of Mb in fresh fish are in the reduced state (Mb-Fe^{2+}), involving deoxyMb and oxyMb content (Thiansilakul *et al.*, 2010). During storage, autoxidation of Mb proceeded, resulting in metMb formation. The result suggested that a rapid oxidation of Mb took place in the early stage of storage (within the first 6 days). During 2 days of storage, the increase in metMb content of yellowtail muscle was accompanied by gradual darkening (Sohn *et al.*, 2005). The formation of metMb negatively affected the apparent color of tuna slices during refrigerated storage.

Among all samples, those without phenolic compounds more likely had the highest metMb formation during storage, regardless of packaging atmosphere. In the presence of phenolic compounds, lowered metMb formation was achieved. AC sample showed a higher %metMb than AT sample during day 3-6, whereas MC sample had a higher %metMb than MT sample during day 3-9 of storage ($P < 0.05$). Thus, tannic acid had a greater efficiency in retardation of Mb oxidation in tuna slices than did caffeic acid. It was noted that MAP induced Mb oxidation of tuna slices. However, MT sample had a lower metMb formation than A sample during day 3-9 of storage ($P < 0.05$). MC sample showed a lower metMb formation than A sample at day 3, followed by slight differences until day 9 of storage. The result suggested a synergistic effect between MAP and tannic acid on retarding metMb formation during storage. Reducing power of phenolic compounds varied upon their type and molecular structure (Medina *et al.*, 2007). Tannic acid more likely reduced ferric (Fe^{3+}) into ferrous (Fe^{2+}), thereby lowering Mb oxidation.

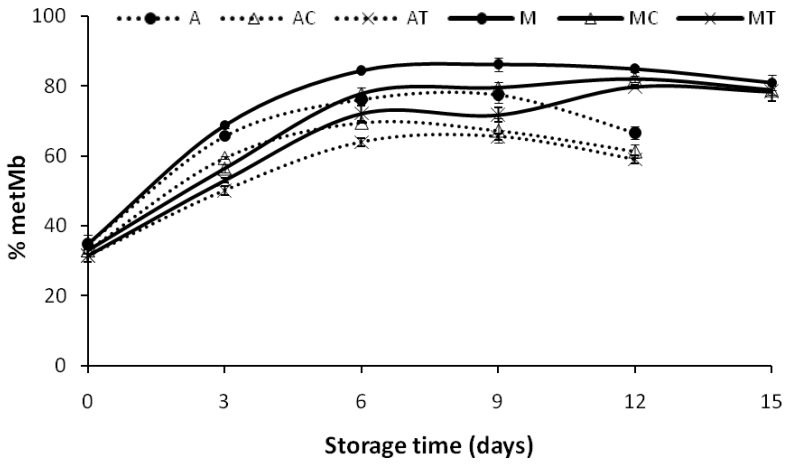


Figure 44. Changes in %metMb of Eastern little tuna slices treated without and with caffeic acid or tannic acid (200 ppm) and stored in air or under MAP ($60\%CO_2/5\%O_2/35\%N_2$) during 15 days of refrigerated storage. Bars represent the standard deviation (n = 3). A : tuna slice stored in air, AC : tuna slice treated with caffeic acid and stored in air, AT : tuna slice treated with tannic acid and stored in air, M : tuna slice stored under MAP, MC : tuna slice treated with caffeic acid and stored under MAP, MT : tuna slice treated with tannic acid and stored under MAP

9.4.2 Changes in redness

Changes in redness (a^*-value) of tuna slices treated without and with phenolic compounds and stored in air or under MAP during storage at 4ºC are shown in Figure 45. Redness of samples kept in air decreased sharply within the first 3 days of storage ($P < 0.05$). Thereafter, slight decreases in redness of AT sample were observed with increasing storage time up to day 9. Nevertheless, negligible changes were observed in A and AC samples ($P > 0.05$). For samples kept under MAP, redness decreased markedly within the first 6 days of storage ($P < 0.05$) and no changes in redness were found until day 15 of storage ($P > 0.05$). Decrease in redness of tuna slices, especially in the early stage of storage, plausibly resulted from the formation of metMb (Figure 44). This led to the development of brown color in slices. The redness of sardine and mackerel muscles decreased when the storage time increased and coincided with the formation of metMb (Chaijan *et al.*, 2005). Notably, samples kept in air

exhibited the increases in redness at day 12 of storage ($P < 0.05$). This was possibly due to the production of nitric oxide from bacteria, which were able to convert metMb to nitrosoMb, the bright red pigment (Gündogdu *et al.*, 2006).

During storage, tuna slices treated with tannic acid and stored under both packaging atmospheres (AT and MT) likely showed the higher redness than other samples. No differences in redness were observed between A and AC samples ($P > 0.05$), while redness of MC sample was higher than of M sample during day 6-9 of storage ($P < 0.05$). The result indicated that tannic acid could retard a loss of redness in tuna slices during storage in air and under MAP. It was noted that caffeic acid was able to retard discoloration of tuna slices kept under MAP but could not inhibit the color changes in samples stored in air. In general, MAP tended to induce discoloration of tuna slices in comparison with air. However, MT sample had a higher redness than A sample during day 3-9 of storage and slight differences were found between MT and AT samples. MAP with high CO_2 and low O_2 contents, which was capable of inhibiting microbial growth and off-odor/flavor development, had a less efficiency in maintaining redness of fish slices (Masniyom *et al.*, 2002). When tannic acid was incorporated, a loss of redness in tuna slices could be prevented, particularly within the first 9 days.

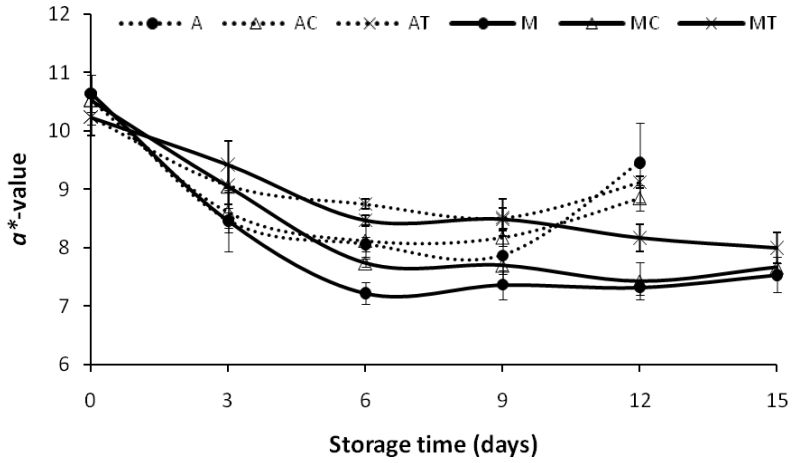


Figure 45. Changes in *a**-value of Eastern little tuna slices treated without and with caffeic acid or tannic acid (200 ppm) and stored in air or under MAP ($60\%CO_2/5\%O_2/35\%N_2$) during 15 days of refrigerated storage. Bars represent the standard deviation ($n = 3$). Key: see Figure 44 caption

9.4.3 Changes in PV

Changes in PV of tuna slices with different treatments during storage at 4ºC are displayed in Figure 46A. For samples kept in air, the marked increases in PV were observed with increasing storage time up to day 6, followed by negligible changes during 6-9 days of storage. Thereafter, a decrease was noticeable at day 12 ($P < 0.05$). For samples kept under MAP, the gradual increases in PV were found with increasing storage time, by which the highest PV at day 12 for M and MC samples and at day 15 for MT sample were observed ($P < 0.05$). Unsaturated fatty acids in fish muscle are oxidized easily during storage, leading to the generation of hydroperoxides (Sohn *et al.*, 2005; Thiansilakul *et al.*, 2010). The rapid formations of hydroperoxide were obtained in tuna slices kept in air, while MAP condition could impede the increase in PV. At day 15, the decrease in PV in M and MC samples was probably due to the decomposition of primary oxidation products.

In the absence of phenolic compounds, tuna slices had the higher PV, compared with samples treated with tannic acid or caffeic acid ($P < 0.05$). Addition of tannic acid could lower the increase in PV in tuna slices more effectively than caffeic acid. Furthermore, MAP could inhibit the increase in PV for all samples treated without and with phenolic compounds (M, MC and MT). During storage, MT sample showed the lowest PV, while A sample had the highest PV, compared with the other samples ($P < 0.05$). These indicated the pronounced impact of phenolic compounds, especially tannic acid, in combination with MAP on retardation of lipid oxidation in tuna slices during storage. Although storing samples in air was able to maintain the redness more effectively than MAP condition, it had the lower ability in suppression of lipid oxidation. The high oxygen levels promote oxidative changes in meat and further negatively affect meat quality (Kim *et al.*, 2010).

9.4.4 Changes in TBARS

TBARS of tuna slices with different treatments during storage at 4ºC are shown in Figure 46B. For samples kept in air, TBARS increased gradually with increasing storage time up to day 6 ($P < 0.05$). Thereafter, sharp increases in TBARS were observed, followed by the decreases at day 12 of storage ($P < 0.05$). For samples stored under MAP, TBARS increased as storage time increased, especially during 9-15 days of storage ($P < 0.05$). MAP condition was

able to lower the increase in TBARS, especially when phenolic compounds were incorporated. TBARS value has been used to determine the lipid oxidation secondary products (Nawar, 1996). The decreases in TBARS at the end of storage might be caused by a loss of low molecular weight decomposition products during the advancement of oxidation (Nawar, 1996).

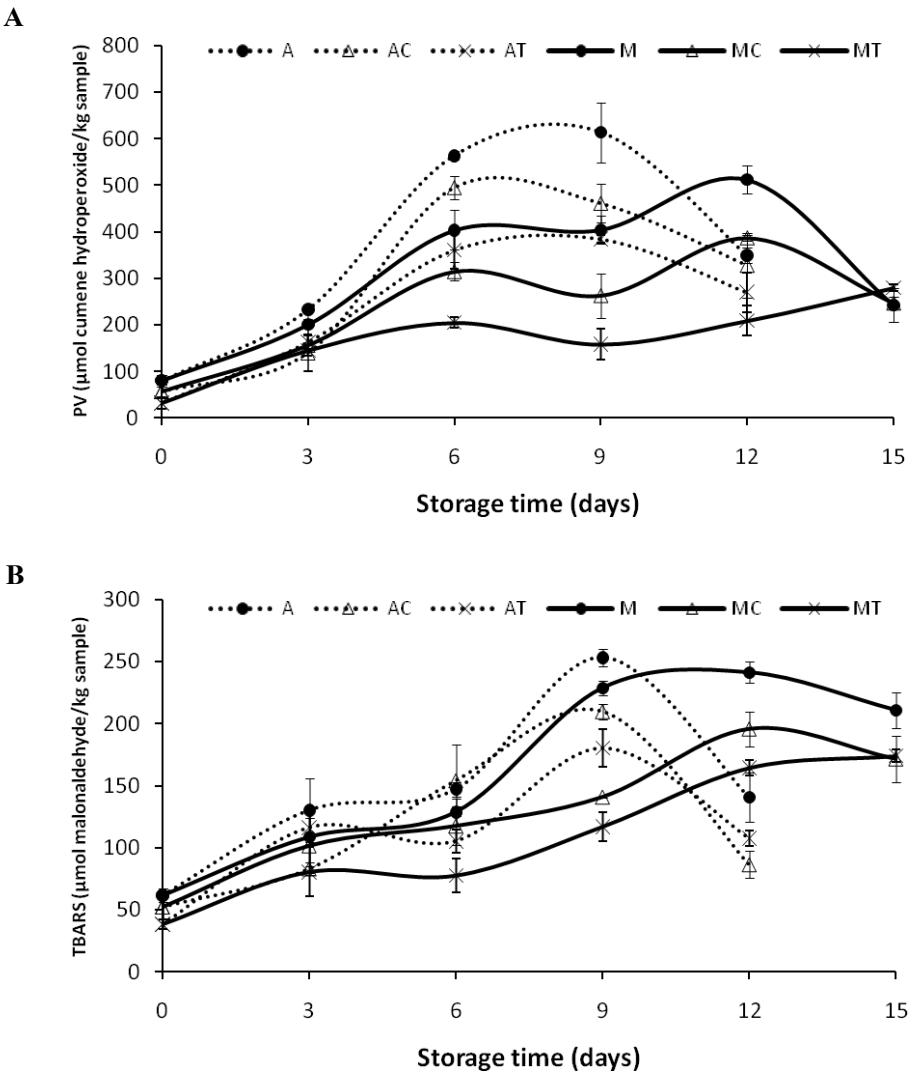


Figure 46. Changes in PV (A) and TBARS (B) of Eastern little tuna slices treated without and with caffeic acid or tannic acid (200 ppm) and stored in air or under MAP ($60\%CO_2/5\%O_2/35\%N_2$) during 15 days of refrigerated storage. Bars represent the standard deviation ($n = 3$). Key: see Figure 44 caption

At day 9, the highest lipid oxidation was observed in A sample, followed by M, AC, AT, MC and MT samples, respectively. In the presence of phenolic compounds, the lower TBARS were observed and tannic acid exhibited a greater antioxidant effect than did caffeic acid. Antioxidative properties of phenolic compounds varied, depending on many factors such as the intrinsic property, the molecular size, the location and orientation in targeted substrate as well as the interactions with other food ingredients (Decker *et al.*, 2005; Kathirvel and Richards, 2009; Lee *et al.*, 2006; Wang *et al.*, 2010). In the presence of tannic acid, a less formation of metMb was found (Figure 44) and related with reduced lipid oxidation in fish samples. MAP with a low level of oxygen and high level of carbon dioxide was capable of retarding the oxidation of lipid, which is normally initiated by oxygen (Nawar, 1996). The result indicated that treatment of phenolic compounds in combination of MAP was effective in preventing lipid oxidation, compared with using phenolic compounds or MAP alone. Therefore, the use of tannic acid together with high CO_2-MAP was selected as the appropriate means to prevent lipid oxidation in tuna slices during storage.

9.4.5 Fatty acid profiles of fresh and refrigerated tuna slices

Fatty acid profiles of fresh tuna slice, A and MT samples stored for 12 days at 4ºC are shown in Table 10. Lipids of fresh tuna slices contained 37.44% SFA, 10.02% MUFA and 52.54% PUFA. Docosahexaenoic acid (DHA, C22:6n3) was found as the most abundant fatty acid (40.91%), followed by palmitic acid (C16:0) (19.72%) and stearic acid (C18:0) (12.93%), respectively. Eicosapentaenoic acid (EPA, C20:5n3) was found at a level of 5.35%. DHA and EPA are beneficial for human wholesomeness, being essential for the development and functionality of certain organs and several physiological and biochemical responses of organisms (Nawar, 1996). It has been suggested that an intake of DHA and EPA ranging from 0.5 to 1.8 g/day significantly reduces subsequent cardiac and all-cause mortality (Kris-Etherton *et al.*, 2003).

After 12 days of refrigerated storage, the decrease in PUFA was remarkably observed in A sample. Only slight decrease in PUFA was found in MT sample, compared with fresh sample. The decrease in PUFA of A sample was coincidental with the increase in SFA contents, indicating the lower degree of unsaturation. DHA and EPA decreased by 2.71% and

6.73%, respectively, for A sample and by 0.32% and 3.36%, respectively, for MT sample. Decreases in PUFA content suggested their susceptibility to oxidation during the extended storage. During storage, triglycerides and phospholipids underwent hydrolysis into free fatty acids, which were prone to oxidation (Thiansilakul *et al.*, 2010). Use of tannic acid in combination with MAP was able to retard the oxidation of lipids in tuna slices, in which n-3 fatty acids were maintained during the extended storage.

Table 10. Fatty acid profile of fresh Eastern little tuna slices, those kept in air (A) and treated with tannic acid (200 ppm) and kept under MAP (60%CO_2/5%O_2/35%N_2) (MT) for 12 days

Fatty acids (g/100 g oil)	Fresh tuna	A sample	MT sample
C12:0	0.06	0.04	0.05
C13:0	0.05	0.06	0.05
C14:0	1.66	1.65	1.67
C15:0	0.89	0.85	0.91
C16:0	19.72	18.45	19.60
C17:0	2.11	2.15	2.15
C18:0	12.93	13.95	13.24
C15:1	0.42	0.55	0.65
C16:1	2.32	2.36	2.04
C17:1	0.46	0.46	0.37
C18:1 *n*-9c	6.52	2.62	2.28
C18:1 *n*-9t	ND	6.06	5.09
C20:1	0.10	ND	ND
C22:1 *n*-9	0.06	ND	ND
C24:1	0.14	ND	ND
C18:2 *n*-6c	1.40	1.62	1.50
C18:3 *n*-6	ND	0.05	ND
C18:3 *n*-3	0.42	0.41	0.43
C20:2	0.25	ND	ND
C20:3 *n*-6	0.16	ND	ND
C20:4 *n*-6	3.95	3.92	4.01
C20:3 *n*-3	0.10	ND	ND
C20:5 *n*-3 (EPA)	5.35	4.99	5.17
C22:6 *n*-3 (DHA)	40.91	39.80	40.78
Saturated fatty acid (SFA)	**10.02**	**12.05**	**10.43**
Monounsaturated fatty acid (MUFA)	**37.42**	**37.15**	**37.67**
Polyunsaturated fatty acid (PUFA)	**52.54**	**50.79**	**51.89**

ND: non-detectable

9.4.6 Microbiological changes

TVC, PBC and LAB of tuna slices with various treatments during storage at 4ºC are depicted in Figure 47A, 47B and 47C, respectively. There were no differences in TVC, PBC and LAB among samples treated without and with phenolic acids, when the same packaging atmosphere was used ($P > 0.05$). TVC of all samples increased as storage time increased ($P < 0.05$). TVC of tuna slices kept in air increased rapidly from an initial value of 10^4 to 10^8 cfu/g within 12 days of storage and were generally higher than that of samples kept under MAP ($P < 0.05$). According to a microbial acceptability limit of 10^7 cfu/g, the shelf-life of tuna slices stored in air and MAP at 4ºC was estimated to be 6 and 12 days, respectively, regardless of treatment of phenolic compounds. This indicated the essential role of CO_2 in retarding the microbial growth. As a result, the shelf-life of tuna slices could be extended during refrigerated storage.

PBC of samples kept in air increased markedly within the first 6 days of storage ($P < 0.05$) without any changes thereafter ($P > 0.05$). At day 3, the lowest PBC was observed in AT sample compared with other samples kept in air. Subsequently, no differences in PBC were obtained. For samples stored under MAP, PBC increased continuously until day 15 of storage ($P < 0.05$). PBC exceeded TVC for all samples at each sampling time, suggesting that psychrophilic bacteria were the dominant microorganisms in Eastern little tuna stored at refrigerated temperature. Similar finding was reported for refrigerated striped catfish and pearl spot (Maqsood and Benjakul, 2010; Ravi Sankar *et al.*, 2008).

LAB of all samples increased with increasing storage time ($P < 0.05$). Samples kept in air had the higher LAB than those stored under MAP within the first 6 days of storage ($P < 0.05$). Thereafter, slight differences in LAB between samples kept in air and under MAP were obtained until the end of storage.

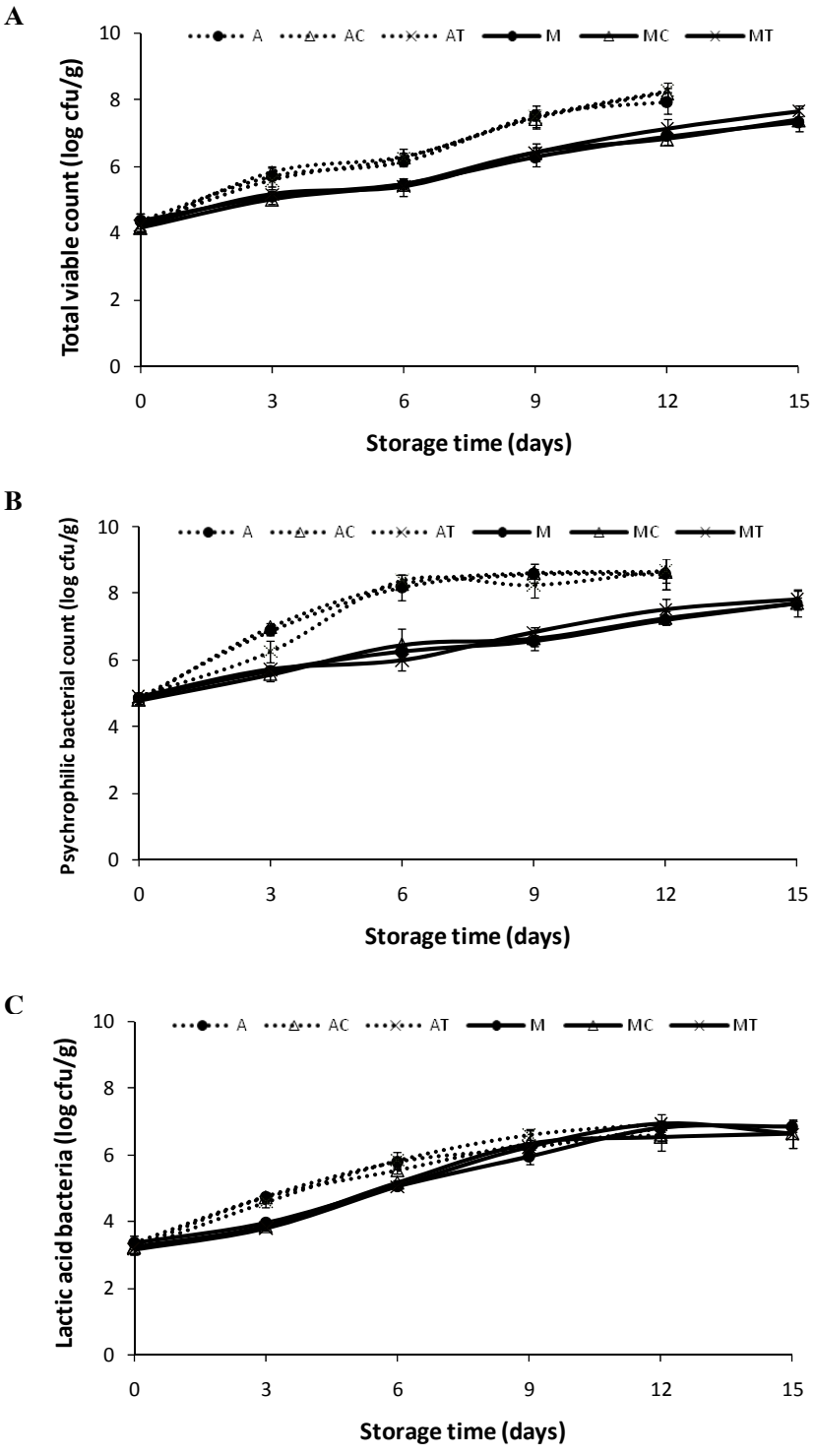


Figure 47. Changes in total viable count (A), psychrophilic bacterial count (B) and lactic acid bacteria (C) of Eastern little tuna slices treated without and with caffeic acid or tannic acid (200 ppm) and stored in air or under MAP ($60\%CO_2/5\%O_2/35\%N_2$) during 15 days of refrigerated storage. Bars represent the standard deviation (n = 3). Key: see Figure 44 caption

MAP was found to be effective in retarding the growth of microorganisms in tuna slices. CO_2 acts as antibacterial agent by displacement of some or all of the O_2 available for bacterial metabolism, thus reducing the growth rate of microorganisms (Sivertsvik *et al.*, 2002). CO_2 at higher concentration was more effective in inhibition of microbial growth in fish slices or fillets (Masniyom *et al.*, 2002; Sivertsvik *et al.*, 2002). CO_2 has been found to exhibit an inhibitory effect mainly against Gram-negative bacteria, which are the major group of microorganism responsible for spoilage of iced stored fish and shellfish (Gram and Huss, 1996; Ravi Sankar *et al.*, 2008; Sivertsvik *et al.*, 2002). Ravi Sankar *et al.* (2008) reported that refrigerated storage of pearl spot under MAP with 60%CO_2/40%O_2 resulted in the inhibition of growth of *Aeromonas* and Enterobacteriaceae and the slowest growth of psychrotrophic bacteria, H_2S-producing bacteria, including *Shewanella putrefaciens* and *Pseudomonas*. MAP condition exhibited a great inhibition effect on mesophilic and psychrophilic bacteria throughout storage. However, a growth of LAB, the Gram-positive microorganism, was more likely promoted under MAP, especially when storage time increased. Different plant polyphenols including tannic acid have been reported for their antimicrobial activity against bacteria causing food-borne disease *in vitro* (Taguri *et al.*, 2004). It was noted that no obvious antimicrobial activity of caffeic acid and tannic acid was found in tuna slices for the present study. Stronger action as antioxidant of phenolic compounds might be associated with the lowered remaining antimicrobial activity. The result was in agreement with Huang *et al.* (1993) who reported that caffeic acid and tannic acid could not inhibit the bacterial growth of catfish during refrigerated storage of 12 days.

9.4.7 Fishy odor development

Fresh tuna slice is generally considered to have high acceptability for consumers. It is susceptible to quality loss caused by both microbiological and chemical reactions. The off-odor developed in fish muscle due to lipid oxidation has been considered as fishy odor (Fu *et al.*, 2009). Development of fishy odor in tuna slices with different treatments during storage at 4ºC is depicted in Figure 48. The intensity of fishy odor in A, M and MC samples increased dramatically within the first 9 days of storage ($P < 0.05$), followed by negligible changes thereafter ($P > 0.05$). For both AC and AT samples, fishy odor intensity increased rapidly as storage time increased. MT sample showed a gradual increase until the end of storage ($P < 0.05$). No differences in fishy

odor intensity were observed among all samples within the first 3 days of storage ($P > 0.05$). During day 9-12, the highest fishy odor intensity was observed in A, AC and M samples, while the lowest value was found in MT sample. Increasing fishy odor intensity was more likely associated with the oxidation of lipids in tuna slices during storage. The lowest lipid oxidation occurred in MT sample (Figure 46A and 46B) related with lowered development of fishy odor. Sohn *et al.* (2005) reported the increase in fishy odor in yellowtail during iced storage and the higher fishy odor intensity was observed in the dark muscle, compared with the ordinary muscle. Addition of tannic acid has been used to prevent lipid oxidation as well as to improve odor of cooked striped catfish stored under MAP (Maqsood and Benjakul, 2010).

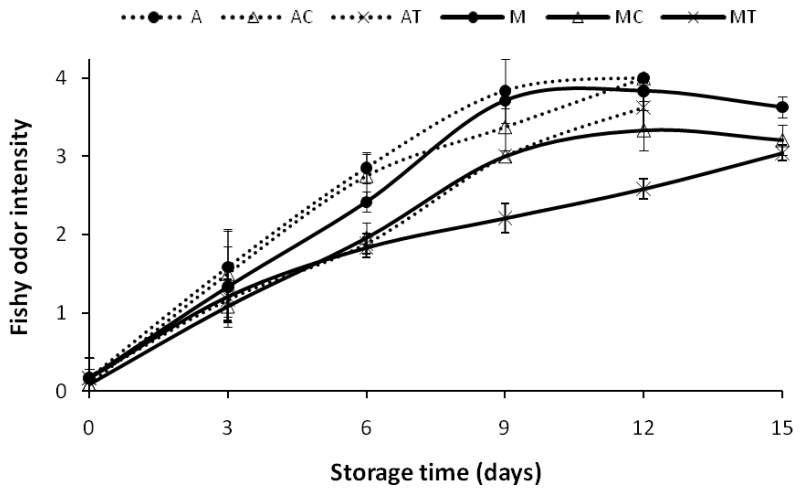


Figure 48. Changes in fishy odor intensity of Eastern little tuna slices treated without and with caffeic acid or tannic acid (200 ppm) and stored in air or under MAP ($60\%CO_2/5\%O_2/35\%N_2$) during 15 days of refrigerated storage. Fishy score ranges from none (score = 0) to strong (score = 4). Bars represent the standard deviation (n = 3). Key: see Figure 44 caption

9.4.8 Likeness

Likeness scores for different attributes including color, odor and overall in different samples during storage at 4ºC are shown in Table 11. The scores of all sensory attributes continuously decreased as storage time increased ($P < 0.05$). During storage, bacterial growth

plays a major role in fish spoilage by generating off-odor, off-flavor and color changes which further reduce acceptability in fish flesh (Masniyom *et al.*, 2002). For the odor likeness, there was no difference in likeness scores among all samples within the first 3 days of storage ($P > 0.05$), except for A sample, which had a lower score than the other samples ($P < 0.05$). Thereafter, the highest likeness score was found in MT, whereas A and AC samples showed the lowest score until the end of storage ($P < 0.05$). With increasing storage time, samples kept under MAP tended to have the higher likeness scores for odor, compared with those stored in air. The result suggested the profound influence of tannic acid on the maintenance of odor likeness, especially under MAP condition. These were in agreement with the rate of lipid oxidation occurred in tuna slices, which was decreased under such a condition.

At day 3, MT sample had the highest color likeness, compared with other samples ($P < 0.05$). M sample showed the lowest color likeness score within the first 9 days of storage. This was concomitant with its highest metMb formation and lowest redness (Figure 44 and 45). MAP decreased the likeness in color of tuna slices. When phenolic acids were incorporated in conjunction with MAP, the likeness scores of tuna slices were greater than those kept in air during day 9-12 of storage. Thus, phenolic compounds combined with MAP were able to lower discoloration of tuna slices with the extended storage.

For overall likeness, samples kept under MAP had the higher likeness scores than those stored in air, especially with increasing storage time. After 3 days of storage, A sample tended to possess the lowest score until the end of storage ($P < 0.05$). The highest overall likeness score was obtained in MT sample throughout the storage of 15 days. Seabass slices kept under CO_2-enriched atmosphere exhibited higher likeness scores for odor and flavor, compared with the untreated sample (Masniyom *et al.*, 2002). As a result, the combination of MAP with phenolic compounds could be used to retard the quality loss of tuna slices. Tannic acid showed the greater potential than caffeic acid in maintaining the likeness of tuna slices, particularly those stored under MAP. Using a score of 5 as the acceptability limit, it was found that tuna slices kept in air and under MAP were acceptable within 3 and 6 days, respectively. Thus, MAP with high CO_2 effectively extended the shelf-life of tuna slices by keeping odor likeness via lowering the development of fishy odor. However, MAP caused discoloration in tuna slices, except for using MAP in combination with tannic acid, in which color of slices was preserved.

Table 11. Likeness scores of Eastern little tuna slices treated without and with caffeic acid and tannic acid during 15 days of refrigerated storage in air or under MAP ($60\%CO_2/5\%O_2/35\%N_2$)

		Storage time (days)					
Attributes	Samples	0	3	6	9	12	15
Odor	A	7.50±0.55Aa*	6.00±0.63Bb	4.00±0.63Cc	1.83±0.75Db	1.17±0.41De	-
	AC	7.83±0.41Aa	6.17±0.41Bab	4.17±0.98Cbc	2.00±0.63Db	1.50±0.55Dde	-
	AT	7.83±0.75Aa	6.50±0.84Bab	5.00±0.63Cabc	2.67±0.52Db	1.83±0.41Ecd	-
	M	7.50±0.55Aa	6.50±0.84Bab	4.83±0.98Cabc	2.17±0.41Db	2.33±0.52Dc	2.00±0.89Dc
	MC	7.83±0.41Aa	6.83±0.75Bab	5.17±0.98Cab	4.17±0.98Da	3.33±0.52DEb	3.17±0.75Eb
	MT	7.83±0.75Aa	7.00±0.63Ba	5.83±0.41Ca	4.67±0.52Da	4.33±0.52Da	4.33±0.52Da
Color	A	7.67±0.52Aa	5.83±0.75Bb	3.83±0.98Cab	3.83±0.75Cab	3.67±0.52Cab	-
	AC	7.33±0.53Aa	5.67±0.82Bb	4.00±1.26Cab	3.67±0.82Cab	3.33±0.52Cb	-
	AT	7.83±0.75Aa	6.50±0.84Bab	4.83±0.75Ca	4.17±0.41Ca	3.33±0.52Db	-
	M	7.67±0.52Aa	5.67±0.82Bb	3.17±0.41Cb	3.17±0.82Cb	3.50±0.55Cab	3.33±0.52Ca
	MC	7.33±0.53Aa	6.33±0.82Bab	3.83±0.75Cab	4.17±0.75Ca	4.00±0.89Cab	3.83±0.98Ca
	MT	7.83±0.75Aa	6.83±0.41Ba	4.17±1.17Cab	4.33±0.82Ca	4.33±1.03CCa	4.17±1.47Ca
Overall	A	7.67±0.52Aa	5.83±0.75Bab	3.83±0.75Cb	2.67±0.82Dd	2.33±0.52Dc	-
	AC	7.50±0.55Aa	5.50±0.84Bb	4.17±0.98Cab	3.00±0.63Dcd	2.67±0.52Dbc	-
	AT	7.83±0.75Aa	6.33±0.52Bab	4.50±0.55Cab	3.50±0.55Dbcd	2.83±0.41Dbc	-
	M	7.67±0.52Aa	5.67±1.03Bab	5.00±1.26Cab	3.67±0.82Dbc	3.17±0.75Dbc	3.17±0.75Da
	MC	7.50±0.55Aa	6.33±1.37ABab	5.33±1.51BCa	4.17±0.98CDab	3.67±1.21Dab	3.50±0.55Da
	MT	7.83±0.75Aa	6.83±1.17Aa	5.50±1.38Ba	4.67±0.82BCa	4.33±1.03BCa	3.83±1.33Ca

* Values with the same uppercase in the same row do not differ significantly ($P > 0.05$). Values with same lowercase in the same column within same attribute do not differ significantly ($P > 0.05$). Key: see Figure 44 caption

9.5 Conclusion

During refrigerated storage, tuna slices underwent discoloration, lipid oxidation and spoilage, leading to a lower acceptance. In the presence of phenolic compounds (200 ppm), metMb formation and lipid oxidation were retarded. Generally, the greater antioxidative activity was found in tannic acid, compared with caffeic acid. Therefore, treatment of tuna slices with tannic acid in combination with MAP ($60\%CO_2/5\%O_2/35\%N_2$) could maintain the quality of tuna slices with the shelf-life of 12 days.

CHAPTER 10

SUMMARY AND FUTURE WORKS

10.1 Summary

1. Seabass which contained the higher contents of polyunsaturated fatty acids and Mb had the higher lipid deterioration and off-odor development, compared with red tilapia, during iced storage.
2. Mb isolated from dark muscle of Eastern little tuna had MW of 15,680 Da, pI 5.25 and transition temperature 60-61ºC. Different color and absorption spectra depended on the forms, oxyMb and metMb. Mb was prone to oxidation and denaturation at acidic pH ($\leq$ 3) and high temperature ($\geq$60ºC) in which metMb form was more stable than oxyMb.
3. Saturated oxygen atmosphere resulted in weakening of heme-globin complex and conformational changes of globin. Autoxidation of oxyMb proceeded rapidly in the presence of oxygen. Vacuum packaging could maintain the redness of tuna fillet, while fillets stored in exposed air or packed in 100% O_2 atmosphere turned to be brown, due to Mb oxidation.
4. The reaction between H_2O_2 and oxyMb/metMb caused the formation of ferrylMb and release of non-heme iron from Mb. Aldehydes were able to oxidize oxyMb and resulted in conformational changes of globin. Hexenal was found to have an impact on Mb cross-linking.
5. Mb mediated discoloration, lipid oxidation as well as off-odor development occurred in washed mince to a greater extent during refrigerated storage, especially at the lower pH. 1-octen-3-ol and hexanal were the major volatile compounds contributing to off-odor in fish mince.
6. Heme proteins, Mb and Hb, present in muscle of all fish species had a close relationship with post-mortem lipid oxidation. Hb, having low hemin

affinity and rapid autoxidation, promoted the greater lipid oxidation than did Mb. Met-form was likely a stronger pro-oxidant than oxy-form.

7. Caffeic acid or gallic acid (200 ppm) had the higher retardation effect against lipid oxidation mediated by Mb and Hb, compared with tannic acid. Treatment of tuna slices with tannic acid in combination with MAP (60%CO_2, 35%N_2 and 5%O_2) could maintain the quality of tuna slices with the shelf-life of 12 days.

10.2 Future works

1. Pro-oxidative activity of Mb and Hb of other fish species should be further studied.
2. Different factors which influence the pro-oxidative activity of heme proteins should be further investigated, in which promising preventive method can be gained.
3. The conformational changes of heme proteins associated with their pro-oxidative property should be elucidated.
4. Selected phenolic compounds and/or CO_2-modified atmosphere packaging should be further studied to prevent the quality loss of fish muscle during storage.

REFERENCES

Akhrem, A.A., Andreyuk, G.M., Kisel, M.A. and Kiselev, P.A. 1989. Hemoglobin conversion to hemichrome under the influence of fatty acids. Biochim. Biophys. Acta. 992: 191-194.

Al-Shaibani, K.A., Price, R.J. and Brown, W.D. 1977. Purification of metmyoglobin reductase from bluefin tuna. J. Food Sci. 42: 1013-1015.

Andersen, H.J. and Skibsted, L.H. 1991. Oxidative stability of frozen pork patties. Effect of light and added salt. J. Food Sci. 56: 1182-1184.

Angelo, A.J.S., Vercellotti, D.J., Jacks, T. and Legendre, M.M. 1996. Lipid oxidation in foods. Crit. Rev. Food Sci. Nutr. 36: 175-224.

Antonini, E. and Brunori, M. 1971. Hemoglobin and myoglobin in their reactions with ligands. North-Holland Publishing Co. Amsterdam.

AOAC. 2000. Official Methods of Analysis. 17th Ed. Association of Official Chemists. Gaithersberg, Maryland.

Apte, S. and Morrissey, P.A. 1987. Effect of haemoglobin and ferritin on lipid oxidation in raw and cooked muscle systems. Food Chem. 25: 127-134.

Aranda IV, R., Worley, C.E., Liu, M., Bitto, E., Cates, M.S., Olson, J.S., Lei, B. and Phillips Jr, G.N. 2007. Bis-methionyl coordination in the crystal structure of the heme-binding domain of the streptococcal cell surface protein Shp. J. Mol. Biol. 374: 374-383.

Ashie, I.N.A., Smith, J.P. and Simpson, B.K. 1996. Spoilage and shelf-life extension of fresh fish and shellfish. Crit. Rev. Food Sci. Technol. 36: 87-121.

Baron, C.P., Bro, R., Skibsted, L.H. and Andersen, H.J. 1997. Direct measurement of lipid peroxidation in oil-in-water emulsions using multiwavelength derivative UV-spectroscopy. J. Agric. Food Chem. 45: 1741-1745.

Baron, C.P., Skibsted, L.H. and Andersen, H.J. 2000. Peroxidation of linoleate at physiological pH: hemichrome formation by substrate binding protects against metmyoglobin activation by hydrogen peroxide. Free Radical Biol. Med. 28: 549-558.

Baron, C.P. and Andersen, H.J. 2002. Myoglobin-induced lipid oxidation: A review. J. Agric. Food Chem. 50: 3887-3897.

Baron, C.P., Skibsted, L.H. and Andersen, H.J. 2002. Concentration effects in myoglobin-catalyzed peroxidation of linoleate. J. Agric. Food Chem. 50: 883-888.

Benjakul, S. and Bauer, F. 2001. Biochemical and physicochemical changes in catfish (*Silurus glanis* Linne) muscle as influenced by different freeze-thaw cycles. Food Chem. 72: 207-217.

Benjakul, S., Visessanguan, W. and Leelapongwattana, K. 2002. Characteristics of muscle from two species of bigeye snapper, *Priacanthus tayenus* and *Priacanthus macracanthus*. J. Food Biochem. 26: 307-326.

Berns, R.S. 2000. Billmeyer and Saltzman's principles of color technology. Wiley. New York.

Binotti, I., Giovenco, S., Giardina, B., Antonini, E., Brunori, M. and Wyman, J. 1971. Studies on the functional properties of fish hemoglobins. II. The oxygen equilibrium of the isolated hemoglobin components from trout blood. Arch. Biochem. Biophys. 142: 274-280.

Bligh, E.G. and Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917.

Bonafe, C.F.S., Matsukuma, A.Y. and Matsuura, M.S.A. 1999. ATP-induced tetramerization and cooperativity in hemoglobin of lower vertebrates. J. Biol. Chem. 274: 1196-1198.

Bontidean, I., Berggren, C., Johansson, G., Csoregi, E., Mattiasson, B., Lloyd, J.R., Jakeman, K.J. and Brown, N.L. 1998. Detection of heavy metal ions at femtomolar levels using protein-based biosensors. Anal. Chem. 70: 4162-4169.

Brantley, R.E., Jr., Smerdon, S.J., Wilkinson, A.J., Singleton, E.W. and Olson, J.S. 1993. The mechanism of autooxidation of myoglobin. J. Biol. Chem. 268: 6995-7010.

Brown, W.D. 1962. The concentration of myoglobin and hemoglobin in tuna flesh. J. Food Sci. 27: 26-28.

Brown, W.D., Albright, M., Watts, D.A., Heyer, B., Spruce, B. and Price, R.J. 1980. Modified atmosphere storage of rockfish (*Sebastes miniatus*) and silver salmon (*Oncorhynchus kisutch*). J. Food Sci. 45: 93-96.

Buege, J.A. and Aust, S.D. 1978. Microsomal lipid peroxidation methods. Methods Enzymol. 52: 302–310.

Bunn, H.F. and Jandl, J.H. 1966. Exchange of heme among hemoglobin molecules. Proceedings of the National Academy of Sciences of the United States of America. 56: 974-978.

Buttery, R.G., Turnbaugh, J.G. and Ling, L.C. 1988. Contribution of volatiles to rice aroma. J. Agric. Food Chem. 36: 1006-1009.

Cannon, J.B., Kuo, F.S., Pasternack, R.F., Wong, N.M. and Muller-Eberhard, U. 1984. Kinetics of the interaction of hemin liposomes with heme binding proteins. Biochemistry. 23: 3715-3721.

Carlsen, C.U., Moller, J.K.S. and Skibsted, L.H. 2005. Heme-iron in lipid oxidation. Coord. Chem. Rev. 249: 485-498.

Castell, C.H., MacLean, J. and Moore, B. 1965. Rancidity in lean fish muscle. IV. Effect of sodium chloride and other salts. J. Fish. Res. Board Can. 22: 929-944.

Chaijan, M., Benjakul, S., Visessanguan, W. and Faustman, C. 2004. Characteristics and gel properties of muscles from sardine (*Sardinella gibbosa*) and mackerel (*Rastrelliger kanagurta*) caught in Thailand. Food Res. Int. 37: 1021-1030.

Chaijan, M., Benjakul, S., Visessanguan, W. and Faustman, C. 2005. Changes of pigments and color in sardine (*Sardinella gibbosa*) and mackerel (*Rastrelliger kanagurta*) muscle during iced storage. Food Chem. 93: 607-617.

Chaijan, M., Benjakul, S., Visessanguan, W. and Faustman, C. 2006. Changes of lipids in sardine (*Sardinella gibbosa*) muscle during iced storage. Food Chem. 99: 83-91.

Chaijan, M., Benjakul, S., Visessanguan, W. and Faustman, C. 2007. Characterisation of myoglobin from sardine (*Sardinella gibbosa*) dark muscle. Food Chem. 100: 156-164.

Chaijan, M. 2008. Review: Lipid and myoglobin oxidations in muscle foods. Songklanakarin J. Sci. Technol. 30: 47-53.

Chan, W.K.M., Faustman, C. and Decker, E.A. 1997a. Oxymyoglobin oxidation as affected by oxidation products of phosphatidylcholine liposomes. J. Food Sci. 62: 709-712.

Chan, W.K.M., Faustman, C., Yin, M. and Decker, E.A. 1997b. Lipid oxidation induced by oxymyoglobin and metmyoglobin with involvement of H_2O_2 and superoxide anion. Meat Sci. 46: 181-190.

Chantachum, S., Benjakul, S. and Sriwirat, N. 2000. Separation and quality of fish oil from precooked and non-precooked tuna heads. Food Chem. 69: 289-294.

Chanthai, S., Ogawa, M., Tamiya, T. and Tsuchiya, T. 1996. Studies on thermal denaturation of fish myoglobins using differential scanning calorimetry, circular dichroism and tryptophan fluorescence. Fish. Sci. 62: 927-932.

Chen, H.M., Muramoto, K. and Yamauchi, F. 1995. Structural analysis of antioxidative peptides from soybean beta-conglycinin. J. Agric. Food Chem. 43: 574-578.

Chen, L.C., Lin, S.B. and Chen, H.H. 2004. Thermal stability and denaturation rate of myoglobin from various species of fish. Fish. Sci. 70: 293-298.

Chen, W.L. and Chow, C.J. 2001. Studies on the physicochemical properties of milkfish myoglobin. J. Food Biochem. 25: 157-174.

Cheng, J.-H. and Ockerman, H.W. 2004. Effect of ascorbic acid with tumbling on lipid oxidation of precooked roast beef. J. Muscle Foods. 15: 83-93.

Chi, E.Y., Krishnan, S., Randolph, T.W. and Carpenter, J.F. 2003. Physical stability of proteins in aqueous solution: Mechanism and driving forces in nonnative protein aggregation. Pharmaceut. Res. 20: 1325-1336.

Chow, C.J., Liu, S.M. and Tsai, M.L. 1997. Characteristics of reaction between carbon monoxide gas and myoglobin in tuna flesh. J. Food Drug Anal. 5: 199-206.

Chow, C.J., Ochiai, Y. and Watabe, S. 2004. Effect of frozen temperature on autoxidation and aggregation of bluefin tuna myoglobin in solution. J. Food Biochem. 28: 123-134.

Clayton, A.H.A. and Sawyer, W.H. 1999. The structure and orientation of class-A amphipathic peptides on a phospholipid bilayer surface. Eur. Biophys. J. 28: 133-141.

Colonna, G., Irace, G., Bismuto, E., Servillo, L. and Balestrieri, C. 1983. Structural and functional aspects of the heart ventricle myoglobin of bluefin tuna. Comp. Biochem. Physiol., A. 76: 481-485.

Conway, E.J. and Byrne, A. 1933. An absorption apparatus for the micro-determination of certain volatile substances: The micro-determination of ammonia. Biochem. J. 27: 419-429.

Cooper, C.E., Jurd, M., Nicholls, P., Wankasi, M.M., Svistunenko, D.A., Reeder, B.J. and Wilson, M.T. 2005. On the formation, nature, stability and biological relevance of the primary reaction intermediates of myoglobins with hydrogen peroxide. Dalton Trans. 21: 3483-3488.

Copeland, R.A. 1994. Protein folding and stability. *In* Methods for Protein Analysis; A Practical Guide to Laboratory Protocols. (Copeland, R. A., ed.). p. 199-216. Chapman & Hall. New York.

Cousin, M.A., Jay, J.M. and Vasavada, P.C. 1992. Psychrotrophic microorganisms. *In* Compendium of methods for the microbiological examination of foods. (Vanderzand, C. and Splittstoesser, D. F., eds.). p. 153-168. American Public Health Association. Washington DC.

Currie, R.W. and Wolfe, F.H. 1977. Evidence for differences in *post-mortem* intramuscular phospholipase activity in several muscle types. Meat Sci. 1: 185–193.

Davies, M.J. 1990. Detection of myoglobin-derived radicals on reaction of metmyoglobin with hydrogen peroxide and other peroxidic compounds. Free Radic. Res. Commun. 10: 361-370.

Davies, M.J. 1991. Identification of a globin free radical in equine myoglobin treated with peroxides. Biochim. Biophys. Acta. 1077: 86-90.

Decker, E.A. and Hultin, H.O. 1990. Factors influencing catalysis of lipid oxidation by the soluble fraction of mackerel muscle. J. Food Sci. 55: 947-950.

Decker, E.A., Warner, K., Richards, M.P. and Shahidi, F. 2005. Measuring antioxidant effectiveness in food. J. Agric. Food Chem. 53: 4303-4310.

DeGray, J.A., Gunther, M.R., Tschirret-Guth, R., de Montellano, P.R.O. and Mason, R.P. 1997. Peroxidation of a specific tryptophan of metmyoglobin by hydrogen peroxide. J. Biol. Chem. 272: 2359-2362.

Dissaraphong, S., Benjakul, S., Visessanguan, W. and Kishimura, H. 2006. The influence of storage conditions of tuna viscera before fermentation on the chemical, physical and microbiological changes in fish sauce during fermentation. Bioresour. Technol. 97: 2032-2040.

Dix, T.A. and Marnett, L.J. 1985. Conversion of linoleic acid hydroperoxide to hydroxy, keto, epoxyhydroxy, and trihydroxy fatty acids by hematin. J. Biol. Chem. 260: 5351-5357.

Dosi, R., Maro, A.D., Chambery, A., Colonna, G., Costantini, S., Geraci, G. and Parente, A. 2006. Characterization and kinetics studies of water buffalo (*Bubalus bubalis*) myoglobin. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 145: 230-238.

Eder, R. 1996. Pigments. *In* Handbook of food analysis. (Nollet, L. M. L., ed.). p. 996-1005. Marcel Dekker, Inc. New York.

Eilert, S.J. 2005. New packaging technologies for the 21st century. Meat Sci. 71: 122-127.

Emborg, J., Laursen, B.G. and Dalgaard, P. 2005. Significant histamine formation in tuna (*Thunnus albacares*) at 2 °C - effect of vacuum- and modified atmosphere-packaging on psychrotolerant bacteria. Int. J. Food Microbiol. 101: 263-279.

Erkan, N., Ozden, O., Alakavuk, D.U., Yildirim, S.Y. and İnugur, M. 2006. Spoilage and shelf life of sardines (*Sardina pilchardus*) packed in modified atmosphere. Eur. Food Res. Technol. 222: 667-673.

Estevez, M., Ventanas, S. and Cava, R. 2007. Oxidation of lipids and proteins in frankfurters with different fatty acid compositions and tocopherol and phenolic contents. Food Chem. 100: 55-63.

Everse, J. and Hsia, N. 1997. The toxicities of native and modified hemoglobins. Free Radical Biol. Med. 22: 1075-1099.

Eymard, S., Baron, C.P. and Jacobsen, C. 2009. Oxidation of lipid and protein in horse mackerel (*Trachurus trachurus*) mince and washed minces during processing and storage. Food Chem. 114: 57-65.

Fago, A., Carratore, V., di Prisco, G., Feuerlein, R.J., Sottrup-Jensen, L. and Weber, R.E. 1995. The cathodic hemoglobin of *Anguilla anguilla*. J. Biol. Chem. 270: 18897-18902.

Faustman, C. and Cassens, R.G. 1990. The biochemical basis for discoloration in fresh meat: A review. J. Muscle Foods. 1: 217-243.

Faustman, C., Liebler, D.C., McClure, T.D. and Sun, Q. 1999. Alpha, beta-unsaturated aldehydes accelerate oxymyoglobin oxidation. J. Agric. Food Chem. 47: 3140-3144.

Faustman, C., Sun, Q., Mancini, R. and Suman, S.P. 2010. Myoglobin and lipid oxidation interactions: Mechanistic bases and control. Meat Sci. 86: 86-94.

Fenton, H.J.H. 1894. Oxidation of tartaric acid in the presence of iron. J. Chem. Soc. 65: 899-910.

Fernandez-Lopez, J., Sayas-Barbera, E., Munoz, T., Sendra, E., Navarro, C. and Perez-Alvarez, J.A. 2008. Effect of packaging conditions on shelf-life of ostrich steaks. Meat Sci. 78: 143-152.

Fisheries Foreign Affairs Division. 2007. Statistics on Fishery Production (online). Ministry of Agriculture and Co-operatives. Available: http://www.fisheries.go.th/it-stat/data_2550/yearbook2007%282550%29/yearbook2007%2038%20T2.1.pdf. (19 March 2010)

Fisheries Foreign Affairs Division. 2008. Statistics on Fishery Production (online). Ministry of Agriculture and Co-operatives. Available: http://www.fisheries.go.th/it-stat/data_2550/yearbook2007%282550%29/yearbook2007%2038%20T2.1.pdf. (12 August 2011)

Foegeding, E.A., Lanier, T.C. and Hultin, H.O. 1996. Characteristics of edible muscle tissues. *In* Food Chemistry. 3rd Ed. (Fennema, O. R., ed.). p. 879-942. Marcel Dekker. New York.

Fomuso, L.B., Corredig, M. and Akoh, C.C. 2002. Effect of emulsifier on oxidation properties of fish oil-based structured lipid emulsions. J. Agric. Food Chem. 50: 2957-2961.

Fosmire, G.J. and Brown, W.D. 1976. Yellowfin tuna (*Thunnus albacares*) myoglobin: characterization and comparative stability. Comp. Biochem. Physiol. 55B: 293-299.

Frankel, E.N., Huang, S.W., Prior, E. and Aeschbach, R. 1996. Evaluation of antioxidant activity of rosemary extracts, carnosol and carnosic acid in bulk vegetable oils and fish oil and their emulsions. J. Sci. Food Agric. 72: 201-208.

Fraser, D.I., Dingle, J.R., Hines, J.A., Nowlan, S.C. and Dyer, W.J. 1967. Nucleotide degradation, monitored by thin-layer chromatography, and associated *post-mortem* changes in relaxed cod muscle. J. Fish. Res. Board Can. 24: 1837-1841.

Fu, X., Xu, S. and Wang, Z. 2009. Kinetics of lipid oxidation and off-odor formation in silver carp mince: The effect of lipoxygenase and hemoglobin. Food Res. Int. 42: 85-90.

Fyhn, U., Fyhn, H., Davis, B., Powers, D., Fink, W. and Garlick, R. 1973. Hemoglobin heterogeneity in Amazonian fishes. Comp. Biochem. Physiol., A: Mol. Integr. Physiol. 62: 39-66.

Gajewski, E. and Dizdaroglu, M. 1990. Hydroxyl radical induced cross-linking of cytosine and tyrosine in nucleohistone. Biochemistry. 29: 977-980.

Galaris, D., Sevanian, A., Cadenas, E. and Hochstein, P. 1990. Ferrylmyoglobin-catalyzed linoleic acid peroxidation. Arch. Biochem. Biophys. 281: 163-169.

Garry, D., Meeson, A., Yan, Z. and Williams, R. 2000. Life without myoglobin. Cell. Mol. Life Sci. 57: 896-898.

George, P. and Stratmann, C.J. 1952. The oxidation of myoglobin to metmyoglobin by oxygen. 2. The relation between the first order rate constant and the partial pressure of oxygen. Biochem. J. 51: 418-425.

George, P. and Stratmann, C.J. 1954. The oxidation of myoglobin to metmyoglobin by oxygen. 3. Kinetic studies in the presence of carbon monoxide, and at different hydrogen-ion concentrations with considerations regarding the stability of oxymyoglobin. Biochem. J. 57: 568–573.

Giddings, G.G. 1974. Reduction of ferrimyoglobin in meat. CRC Crit. Rev. Food Technol. 5: 143-173.

Gomez-Basauri, J.V. and Regenstein, J.M. 1992. Vacuum packaging, ascorbic acid and frozen storage effects on heme and nonheme iron content of mackerel. J. Food Sci. 57: 1337-1339.

Gordon, M.H. 2001. The development of oxidative rancidity in foods. *In* Antioxidants in Food: Practical Applications. (Pokorny, J. *et al.*, eds.). p. 6–21. Woodhead Publishing. USA.

Gorelik, S. and Kanner, J. 2001. Oxymyoglobin oxidation and membrane lipid peroxidation initiated by iron redox cycle: prevention of oxidation by enzymic and nonenzymic antioxidants. J. Agric. Food Chem. 49: 5945-5950.

Gotoh, T. and Shikama, K. 1976. Generation of the superoxide radical during autoxidation of oxymyoglobin. J. Biochem. 80: 397-399.

Gram, L. and Huss, H.H. 1996. Microbiological spoilage of fish and fish products. Int. J. Food Microbiol. 33: 121-137.

Greene, B.E. and Price, L.G. 1975. Oxidation-induced color and flavor changes in meat. J. Agric. Food Chem. 23: 164-167.

Grunwald, E.W. and Richards, M.P. 2006a. Studies with myoglobin variants indicate that released hemin is the primary promoter of lipid oxidation in washed fish muscle. J. Agric. Food Chem. 54: 4452-4460.

Grunwald, E.W. and Richards, M.P. 2006b. Mechanisms of heme protein-mediated lipid oxidation using hemoglobin and myoglobin variants in raw and heated washed muscle. J. Agric. Food Chem. 54: 8271-8280.

Guillen, M.D. and Cabo, N. 1997. Infrared spectroscopy in the study of edible oils and fats. J. Sci. Food Agric. 75: 1-11.

Guillen, M.D. and Cabo, N. 2004. Study of the effects of smoke flavourings on the oxidative stability of the lipids of pork adipose tissue by means of Fourier transform infrared spectroscopy. Meat Sci. 66: 647-657.

Gündogdu, A., Karahan, A. and Çakmakç, M. 2006. Production of nitric oxide (NO) by lactic acid bacteria isolated from fermented products. Eur. Food Res. Technol. 223: 35-38.

Gutteridge, J.M. and Halliwell, B. 1990. The measurement and mechanism of lipid peroxidation in biological systems. Trends Biochem. Sci. 15: 129-135.

Haard, N.F. 1992. Biochemistry and chemistry of color and color change in seafoods. *In* Advances in Seafood Biochemistry. (Flick, G. J. and Martin, R. E., eds.). p. 312-319. Technomic Publishing Co., Inc. USA.

Hajeb, P., Jinap, S., Ismail, A., Fatimah, A.B., Jamilah, B. and Abdul Rahim, M. 2009. Assessment of mercury level in commonly consumed marine fishes in Malaysia. Food Control. 20: 79-84.

Hanan, T. and Shaklai, N. 1995. Peroxidative interaction of myoglobin and myosin. Eur. J. Biochem. 233: 930-936.

Harel, S. and Kanner, J. 1985. Muscle membranal lipid peroxidation initiated by hydrogen peroxide-activated metmyoglobin. J. Agric. Food Chem. 33: 1188-1192.

Hargrove, M.S., Singleton, E.W., Quillin, M.L., Ortiz, L.A., Phillips, G., Olson, J.S. and Mathews, A.J. 1994. His^{64} (E7) Tyr apomyoglobin as a reagent for measuring rates of hemin dissociation. J. Biol. Chem. 269: 4207-4214.

Hasegawa, H. 1987. Determination of total viable count (TVC). *In* Laboratory Manual on Analytical Methods and Procedures for Fish and Fish Products, Part E. (Hasegawa, H., ed.). p. 2.1-2.3. Marine Fisheries Research Department, Singapore.

Hazell, T. 1982. Iron and zinc compounds in the muscle meats of beef, lamb, pork and chicken. J. Sci. Food Agric. 33: 1049-1056.

He, K., Nukada, H., Urakami, T. and Murphy, M.P. 2003. Antioxidant and pro-oxidant properties of pyrroloquinoline quinone (PQQ): implications for its function in biological systems. Biochem. Pharmacol. 65: 67-74.

Heu, M.S., Park, S.H., Kim, H.S., Kim, H.J., Han, B.W., Ji, S.G., Kim, J.G., Yoon, M.S. and Kim, J.S. 2008. Improvement on fish odor of extracts from salmon frame soaked in soybean milk. J. Korean Soc. Food Sci. Nutr. 37: 223-230.

Hilse, K. and Braunitzer, G. 1968. Hemoglobin. XVI. Amino acid sequence of the alpha-chain of both main components of carp hemoglobin. Hoppe-Seyler's Z. Physiol. Chem. 349: 433-450.

Hogg, N., Rice-Evans, C., Darley-Usmar, V., Wilson, M.T., Paganga, G. and Bourne, L. 1994. The role of lipid hydroperoxides in the myoglobin-dependent oxidation of LDL. Arch. Biochem. Biophys. 314: 39-44.

Hu, M., Julian McClements, D. and Decker, E.A. 2004. Impact of chelators on the oxidative stability of whey protein isolate-stabilized oil-in-water emulsions containing [omega]-3 fatty acids. Food Chem. 88: 57-62.

Huang, S.W. and Frankel, E.N. 1997. Antioxiciant activity of tea catechins in different lipid systems. J. Agric. Food Chem. 45: 3033-3038.

Huang, Y.W., Low, I., Chung, K.-T. and Huang, C.Y. 1993. Effects of tannic acid, gallic acid and propyl gallate on storage life of catfish. Proceedings of Tropical and Subtropical Fisheries Technological Conference of the Americas. University of Florida, Gainesville, Florida, USA. 234-239.

Hunt, M.C., Mancini, R.A., Hachmeister, K.A., Kropf, D.H., Merriman, M., Lduca, G. and Milliken, G. 2004. Carbon monoxide in modified atmosphere packaging affects color, shelf life, and microorganisms of beef steaks and ground beef. J. Food Sci. 69: 45-52.

Huss, H.H. 1995. Quality and quality changes in fresh fish. *In* FAO Fishery Technical Paper. p. 348. Rome.

Hwang, K.T. and Regenstein, J. 1993. Characteristics of mackerel mince lipid hydrolysis. J. Food Sci. 58: 79-83.

Iglesias, J. and Medina, I. 2008. Solid-phase microextraction method for the determination of volatile compounds associated to oxidation of fish muscle. J. Chromatogr. 1192: 9-16.

Ivana, S., Simona, T., Pier Luigi, M., Rita, C. and Gaetano, I. 2000. The effect of tryptophanyl substitution on folding and structure of myoglobin. Eur. J. Biochem. 267: 3937-3945.

Jadhav, S.J., Nimbalkar, S.S., Kulkarni, A.D. and Madhavi, D.L. 1995. Lipid oxidation in biological and food systems. *In* Food Science and Technology p. 5-64. Marcel Dekker. New York.

Jensen, F.B. 2004. Red blood cell pH, the Bohr effect, and other oxygenation-linked phenomena in blood O_2 and CO_2 transport. Acta Physiol. Scand. 182: 215-227.

Josephson, D.B., Lindsay, R.C. and Stuiber, D.A. 1987. Enzymic hydroperoxide initiated effects in fresh fish. J. Food Sci. 52: 596-600.

Judge, M., Aberle, E., Forrest, J., Hedrick, H. and Merkel, R. 1994. Principles of Meat Science. 3rd Ed. Kendall/Hunt Publishing Co. Dubuque, IA.

Kanner, J. and Harel, S. 1985. Initiation of membranal lipid peroxidation by activated metmyoglobin and methemoglobin. Arch. Biochem. Biophys. 237: 314-321.

Kanner, J., Shegalovich, I., Harel, S. and Hazan, B. 1988. Muscle lipid peroxidation dependent on oxygen and free metal ions. J. Agric. Food Chem. 36: 409-412.

Kanner, J. 1994. Oxidative processes in meat and meat products: quality implications. Meat Sci. 36: 169-189.

Kasahara, K. and Osawa, C. 1998. Combination effects of spices on masking of odor in boiled sardine. Fish. Sci. 64: 415-418.

Kathirvel, P. and Richards, M.P. 2009. Mechanisms by which flavonol aglycones inhibit lipid oxidation better than glycosylated flavonols in comminuted muscle tissue. Food Chem. 117: 75-82.

Ke, P.J., Ackman, R.G. and Linke, B.A. 1975. Autoxidation of polyunsaturated fatty compounds in mackerel oil: formation of 2,4,7-decatrienals. J. Am. Oil Chem. Soc. 53: 349-353.

Kendrick, J. and Watts, B.M. 1969. Acceleration and inhibition of lipid oxidation by heme compounds. Lipids. 4: 454-458.

Kikugawa, K., Kato, T. and Hayasaka, A. 1991. Formation of dityrosine and other fluorescent amino acids by reaction of amino acids with lipid hydroperoxides. Lipids. 26: 922-929.

Kim, Y.H., Huff-Lonergan, E., Sebranek, J.G. and Lonergan, S.M. 2010. High-oxygen modified atmosphere packaging system induces lipid and myoglobin oxidation and protein polymerization. Meat Sci. 85: 759-767.

Kitahara, Y., Matsuoka, A., Kobayashi, N. and Shikama, K. 1990. Autoxidation of myoglobin from bigeye tuna fish (*Thunnus obesus*). Biochim. Biophys. Acta. 1038: 23-28.

Kris-Etherton, P.M., Harris, W.S. and Appel, L.J. 2003. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. Atertio. Thromb. Vasc. Biol. 23: e20-e30.

Kristensen, L. and Andersen, H.J. 1997. Effect of heat denaturation on the pro-oxidative activity of metmyoglobin in linoleic acid emulsions. J. Agric. Food Chem. 45: 7-13.

Krzywicki, K. 1982. The determination of haem pigments in meat. Meat Sci. 7: 29-36.

Kyrana, V.R. and Lougovois, V.P. 2002. Sensory, chemical and microbiological assessment of farm-raised European sea bass (*Dicentrarchus labrax*) stored in melting ice. Int. J. Food Sci. Technol. 37: 319-328.

Labuza, T.P., McNally, L., Gallagher, D., Hawkes, J. and Hurtado, F. 1972. Stability of intermediate moisture foods. 1. Lipid Oxidation. J. Food Sci. 37: 154-159.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Lanier, T.C., Carpenter, J.A., Toledo, R.T. and Reagan, J.O. 1978. Metmyoglobin reduction in beef systems as affected by aerobic, anaerobic and carbon monoxide-containing environments. J. Food Sci. 43: 1788-1792.

Lee, B.J., Hendricks, D.G. and Cornforth, D.P. 1999. A comparison of carnosine and ascorbic acid on color and lipid stability in a ground beef pattie model system. Meat Sci. 51: 245-253.

Lee, C.H., Krueger, C.G., Reed, J.D. and Richards, M.P. 2006. Inhibition of hemoglobin-mediated lipid oxidation in washed fish muscle by cranberry components. Food Chem. 99: 591-599.

Lee, S., Joo, S.T., Alderton, A.L., Hill, D.W. and Faustman, C. 2003a. Oxymyoglobin and lipid oxidation in yellowfin tuna (*Thunnus albacares*) loins. J. Food Sci. 68: 1664-1668.

Lee, S., Phillips, A.L., Liebler, D.C. and Faustman, C. 2003b. Porcine oxymyoglobin and lipid oxidation in *vitro*. Meat Sci. 63: 241-247.

Libondi, T., Ragone, R., Vincenti, D., Stiuso, P., Auricchio, G. and Colonna, G. 1994. In vitro cross-linking of calf lens alpha-crystallin by malondialdehyde. Int. J. Peptide Protein Res. 44: 342-347.

Lindahl, G., Lundstrъm, K. and Tornberg, E. 2001. Contribution of pigment content, myoglobin forms and internal reflectance to the colour of pork loin and ham from pure breed pigs. Meat Sci. 59: 141-151.

Liong, E.C., Dou, Y., Scott, E.E., Olson, J.S. and Phillips Jr, G.N. 2001. Waterproofing the heme pocket. Role of proximal amino acid side chains in preventing hemin loss from myoglobin. J. Biol. Chem. 276: 9093-9100.

Livingston, D.J. and Brown, W.D. 1981. The chemistry of myoglobin and its reactions (Meat pigments, food quality indices). Food Technol. 35: 238-252.

Lowry, R.R. and Tinsley, I.J. 1976. Rapid colorimetric determination of free fatty acids. J. Am. Oil Chem. Soc. 53: 470-472.

Lugasi, A., Losada, V., Hóvári, J., Lebovics, V., Jakóczi, I. and Aubourg, S. 2007. Effect of pre-soaking whole pelagic fish in a plant extract on sensory and biochemical changes during subsequent frozen storage. LWT-Food Sci. Technol. 40: 930-936.

Lund, M.N., Lametsch, R., Hviid, M.S., Jensen, O.N. and Skibsted, L.H. 2007. High-oxygen packaging atmosphere influences protein oxidation and tenderness of porcine longissimus dorsi during chill storage. Meat Sci. 77: 295-303.

Luno, M., Roncales, P., Djenane, D. and Beltran, J.A. 2000. Beef shelf life in low O_2 and high CO_2 atmospheres containing different low CO concentrations. Meat Sci. 55: 413-419.

Lynch, M.P. and Faustman, C. 2000. Effect of aldehyde lipid oxidation products on myoglobin. J. Agric. Food Chem. 48: 600-604.

Maheswarappa, N.B., Faustman, C., Tatiyaborworntham, N., Yin, S., Ramanathan, R. and Mancini, R.A. 2009. Mass spectrometric characterization and redox instability of turkey and chicken myoglobins as induced by unsaturated aldehydes. J. Agric. Food Chem. 57: 8668-8676.

Mancini, R.A. and Hunt, M.C. 2005. Current research in meat color: A review. Meat Sci. 71: 100-121.

Maqsood, S. and Benjakul, S. 2010. Synergistic effect of tannic acid and modified atmospheric packaging on the prevention of lipid oxidation and quality losses of refrigerated striped catfish slices. Food Chem. 121: 29-38.

Maqsood, S. and Benjakul, S. 2011. Comparative studies on molecular changes and pro-oxidative activity of haemoglobin from different fish species as influenced by pH. Food Chem. 124: 875-883.

Masniyom, P., Benjakul, S. and Visessanguan, W. 2002. Shelf-life extension of refrigerated seabass slices under modified atmosphere packaging. J. Sci. Food Agric. 82: 873-880.

Matsuura, F. and Hashimoto, K. 1954. Chemical studies on the red muscle ("chiai") of fishes. II. Determinations of content of hemoglobin, myoglobin and cytochrome c in the muscle of fishes. Bull. Jap. Soc. Sci. Fish. 20: 308-312.

McGill, A.S., Hardy, R. and Gunstone, F.D. 1977. Further analysis of the volatile components of frozen cold stored cod and the influence of these on flavour. J. Sci. Food Agric. 28: 200-205.

Medina, I., Gallardo, J.M., González, M.J., Lois, S. and Hedges, N. 2007. Effect of molecular structure of phenolic families as hydroxycinnamic acids and catechins on their antioxidant effectiveness in minced fish muscle. J. Agric. Food Chem. 55: 3889-3895.

Mei, L., McClements, D.J., Wu, J. and Decker, E.A. 1998. Iron-catalyzed lipid oxidation in emulsion as affected by surfactant, pH and NaCl. Food Chem. 61: 307-312.

Meilgaard, M., Civille, G.V. and Carr, B.T. 2006. Sensory evaluation techniques. 4 Ed. CRC press. Boca Raton, Florida.

Mikkelsen, A. and Skibsted, L.H. 1995. Acid-catalysed reduction of ferrylmyoglobin: product distribution and kinetics of autoreduction and reduction by NADH. Z Lebensm Unters Forsch. 200: 171-177.

Milo, C. and Grosch, W. 1993. Changes in the odorants of boiled trout (Salmo fario) as affected by the storage of the raw material. J. Agric. Food Chem. 41: 2076-2081.

Min, B., Cordray, J.C. and Ahn, D.U. 2010. Effect of NaCl, myoglobin, Fe(II), and Fe(III) on lipid oxidation of raw and cooked chicken breast and beef loin. J. Agric. Food Chem. 58: 600-605.

Misumi, Y., Terui, N. and Yamamoto, Y. 2002. Structural characterization of non-native states of sperm whale myoglobin in aqueous ethanol or 2, 2, 2-trifluoroethanol media. BBA-Proteins and Proteomics. 1601: 75-84.

Mitsuda, H., Yasumoto, K. and Iwami, K. 1966. Antioxidative action of indole compounds during the autoxidation of linoleic acid. Eiyo to Shokuryo. 19: 210-214.

Monahan, F., Crackel, R., Gray, J., Buckley, D. and Morrissey, P. 1993. Catalysis of lipid oxidation in muscle model systems by haem and inorganic iron. Meat Sci. 34: 95-106.

Monahan, F.J., Skibsted, L.H. and Andersen, M.L. 2005. Mechanism of oxymyoglobin oxidation in the presence of oxidizing lipids in bovine muscle. J. Agric. Food Chem. 53: 5734-5738.

Morey, K.S., Hansen, S.P. and Brown, W.D. 1973. Reaction of hydrogen peroxide with myoglobins. J. Food Sci. 38: 1104-1107.

Morrissey, P.A., Sheehy, P.J.A., Galvin, K., Kerry, J.P. and Buckley, D.J. 1998. Lipid stability in meat and meat products. Meat Sci. 49: 73-86.

Nakamura, Y. and Nishida, T. 1971. Effect of hemoglobin concentration on the oxidation of linoleic acid. J. Lipid Res. 12: 149-154.

Nakayama, T., Osawa, T., Mendosa, E.N.T., Laurena, A.C. and Kawakishi, S. 1994. Comparative study of antioxidative assays of plant materials. *In* Posthavest biochemistry of plant food-materials in the tropics. (Uritani, I. *et al.*, eds.). p. 241-251. Japan Scientific Societies Press. Japan.

Nambudiry, D.D. 1980. Lipid oxidation in fatty fish: the effect of salt content in the meat. J. Food Sci. Technol. 17: 176-178.

Namiki, M. 1990. Antioxidants/antimutagens in food. Crit. Rev. Food Sci. Nutr. 29: 273-300.

Nawar, W.W. 1996. Lipids. *In* Food Chemistry. 3rd Ed. (Fennema, O. R., ed.). p. 225-319. Marcel Dekker. New York.

Newman, E.S., Rice-Evans, C.A. and Davies, M.J. 1991. Identification of initiating agents in myoglobin-induced lipid peroxidation. Biochem. Biophys. Res. Commun. 179: 1414-1419.

Nygaard, T.K., Blouin, G.C., Liu, M., Fukumura, M., Olson, J.S., Fabian, M., Dooley, D.M. and Lei, B. 2006. The mechanism of direct heme transfer from the streptococcal cell surface protein Shp to HtsA of the HtsABC transporter. J. Biol. Chem. 281: 20761.

O'Grady, M.N., Monahan, F.J., Burke, R.M. and Allen, P. 2000. The effect of oxygen level and exogenous [alpha]-tocopherol on the oxidative stability of minced beef in modified atmosphere packs. Meat Sci. 55: 39-45.

O'Grady, M.N., Monahan, F.J. and Brunton, N.P. 2001. Oxymyoglobin oxidation and lipid oxidation in bovine muscle-mechanistic studies. J. Food Sci. 66: 386-392.

Ohshima, T., Wada, S. and Koizumi, C. 1988. Influences of heme pigment, non-heme iron, and nitrite on lipid oxidation in cooked mackerel meat. Nippon Suisan Gakk. 54: 2165-2171.

Ordonez, J.A., De Pablo, B., Perez de Castro, B., Asensio, M.A. and Sanz, B. 1991. Selected chemical and microbiological changes in refrigerated pork stored in carbon dioxide and oxygen enriched atmospheres. J. Agric. Food Chem. 39: 668-672.

Osawa, Y. and Williams, M.S. 1996. Covalent crosslinking of the heme prosthetic group to myoglobin by H_2O_2: toxicological implications. Free Radical Biol. Med. 21: 35-41.

Osborn, H.T. and Akoh, C.C. 2004. Effect of emulsifier type, droplet size, and oil concentration on lipid oxidation in structured lipid-based oil-in-water emulsions. Food Chem. 84: 451-456.

Pace, C.N., Trevino, S., Prabhakaran, E. and Scholtz, J.M. 2004. Protein structure, stability and solubility in water and other solvents. Phil. Trans. R. Soc. Lond. B. 359: 1225-1235.

Pacheco-Aguilar, R., Lugo-Sanchez, M.E. and Robles-Burgueno, M.R. 2000. Postmortem biochemical and functional characteristic of monterey sardine muscle stored at 0 °C. J. Food Sci. 65: 40-47.

Pantazi, D., Papavergou, A., Pournis, N., Kontominas, M.G. and Savvaidis, I.N. 2008. Shelf-life of chilled fresh Mediterranean swordfish (*Xiphias gladius*) stored under various packaging conditions: microbiological, biochemical and sensory attributes. Food Microbiol. 25: 136-143.

Parr, A.J. and Bolwell, G.P. 2000. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile J. Sci. Food Agric. 80: 985-1012.

Pawliszyn, J., Pawliszyn, B. and Pawliszyn, M. 1997. Solid phase microextraction (SPME). Chem. Educator. 2: 1-7.

Pazos, M., Lois, S., Torres, J.L. and Medina, I. 2006. Inhibition of hemoglobin- and iron-promoted oxidation in fish microsomes by natural phenolics. J. Agric. Food Chem. 54: 4417-4423.

Pearson, A.M. and Young, R.B. 1989. Sarcoplasmic proteins. *In* Muscle and Meat Biochemistry. (Pearson, A. M. and Young, R. B., eds.). p. 329-333. Academic Press, Inc.

Pegg, R.B. and Shahidi, F. 1997. Unraveling the chemical identity of meat pigments. Crit. Rev. Food Sci. Nutr. 37: 561-589.

Perutz, M.F. 1990. Mechanisms regulating the reactions of human hemoglobin with oxygen and carbon monoxide. Annu. Rev. Physiol. 52: 1-26.

Phillips Jr, G.N., Teodoro, M.L., Li, T., Smith, B. and Olson, J.S. 1999. Bound CO is a molecular probe of electrostatic potential in the distal pocket of myoglobin. J. Phys. Chem. B. 103: 8817-8829.

Pivarnik, L.F., Faustman, C., Rossi, S., Suman, S.P., Palmer, C., Richard, N.L., Ellis, P.C. and DiLiberti, M. 2011. Quality assessment of filtered smoked yellowfin tuna (*Thunnus albacares*) steaks. J. Food Sci. 76: S369-S379.

Prasad, M., Engelman, R., Jones, R. and Das, D. 1989. Effects of oxyradicals on oxymyoglobin. Deoxygenation, haem removal and iron release. Biochem. J. 263: 731-736.

Pratt, D.E. and Hudson, B.J.F. 1990. Natural antioxidants not exploited commercially. *In* Food Antioxidants. (Hudson, B. J. F., ed.). p. 171-179. Elsevier. Amsterdam, Netherlands.

Prigent, S.V.E., Voragen, A.G.J., Visser, A.J.W.G., van Koningsveld, G.A. and Gruppen, H. 2007. Covalent interactions between proteins and oxidation products of caffeoylquinic acid (chlorogenic acid). J. Sci. Food Agric. 87: 2502-2510.

Rao, S.I., Wilks, A., Hamberg, M. and Ortiz de Montellano, P.R. 1994. The lipoxygenase activity of myoglobin. Oxidation of linoleic acid by the ferryl oxygen rather than protein radical. J. Biol. Chem. 269: 7210-7216.

Ravi Sankar, C.N., Lalitha, K.V., Jose, L., Manju, S. and Gopal, T.K.S. 2008. Effect of packaging atmosphere on the microbial attributes of pearlspot (*Etroplus suratensis* Bloch) stored at 0-2 °C. Food Microbiol. 25: 518-528.

Rawdkuen, S., Jongjareonrak, A., Benjakul, S. and Chaijan, M. 2008. Discoloration and lipid deterioration of farmed giant catfish (*Pangasianodon gigas*) muscle during refrigerated storage. J. Food Sci. 73: C179-C184.

Reeder, B.J. and Wilson, M.T. 1998. Mechanism of reaction of myoglobin with the lipid hydroperoxide hydroperoxyoctadecadienoic acid. Biochem. J. 330: 1317-1323.

Renerre, M., Anton, M. and Gatellier, P. 1992. Autoxidation of purified myoglobin from two bovine muscles. Meat Sci. 32: 331-342.

Rice, R.H., Lee, Y.M. and Brown, W.D. 1983. Interactions of heme proteins with hydrogen peroxide: protein crosslinking and covalent binding of benzo[a]pyrene and 17 beta-estradiol. Arch. Biochem. Biophys. 221: 417-427.

Richards, M.P. and Hultin, H.O. 2000. Effect of pH on lipid oxidation using trout hemolysate as a catalyst: A possible role for deoxyhemoglobin. J. Agric. Food Chem. 48: 3141-3147.

Richards, M.P. and Hultin, H.O. 2002. Contributions of blood and blood components to lipid oxidation in fish muscle. J. Agric. Food Chem. 50: 555-564.

Richards, M.P., Modra, A.M. and Li, R. 2002a. Role of deoxyhemoglobin in lipid oxidation of washed cod muscle mediated by trout, poultry and beef hemoglobins. Meat Sci. 62: 157-163.

Richards, M.P., stdal, H. and Andersen, H.J. 2002b. Deoxyhemoglobin-mediated lipid oxidation in washed fish muscle. J. Agric. Food Chem. 50: 1278-1283.

Richards, M.P. and Dettmann, M.A. 2003. Comparative analysis of different hemoglobins: autoxidation, reaction with peroxide, and lipid oxidation. J. Agric. Food Chem. 51: 3886-3891.

Richards, M.P. and Li, R. 2004. Effects of released iron, lipid peroxides, and ascorbate in trout hemoglobin-mediated lipid oxidation of washed cod muscle. J. Agric. Food Chem. 52: 4323-4329.

Richards, M.P., Dettmann, M.A. and Grunwald, E.W. 2005. Pro-oxidative characteristics of trout hemoglobin and myoglobin: A role for released heme in oxidation of lipids. J. Agric. Food Chem. 53: 10231-10238.

Richards, M.P., Cai, H. and Grunwald, E.W. 2009. Phenylalanine substitution at site B10 (L29F) inhibits metmyoglobin formation and myoglobin-mediated lipid oxidation in washed fish muscle: mechanistic implications. J. Agric. Food Chem. 57: 7997-8002.

Richards, M.P. 2010. Heme proteins and oxidation in fresh and processed meats. *In* Oxidation in foods and beverages and antioxidant applications. (Decker, E. and Elias, R., eds.). p. 90. Woodhead Publishing. Cambridge.

Robards, K., Prenzler, P.D., Tucker, G., Swatsitang, P. and Glover, W. 1999. Phenolics compounds and their role in oxidative processes in fruits. Food Chem. 66: 401-436.

Robertson, G.L. 2006. Food packaging: principles and practice. CRC Press. Boca Raton, Florida.

Robinson, S., Dang, T., Dringen, R. and Bishop, G. 2009. Hemin toxicity: a preventable source of brain damage following hemorrhagic stroke. Redox Report. 14: 228-235.

Romero, F., Ordonez, I., Arduini, A. and Cadenas, E. 1992. The reactivity of thiols and disulfides with different redox states of myoglobin. Redox and addition reactions and formation of thiyl radical intermediates. J. Biol. Chem. 267: 1680-1688.

Roth, B., Foss, A. and Imsland, A.K. 2009. Relationship between muscle pH and flesh color of Atlantic halibut. J. Food Sci. 74: S123-S125.

Saeed, S. and Howell, N.K. 2002. Effect of lipid oxidation and frozen storage on muscle proteins of Atlantic mackerel (*Scomber scombrus*). J. Sci. Food Agric. 82: 579-586.

Sanchez-Alonso, I., Borderias, J., Larsson, K. and Undeland, I. 2007. Inhibition of hemoglobin-mediated oxidation of regular and lipid-fortified washed cod mince by a white grape dietary fiber. J. Agric. Food Chem. 55: 5299-5305.

Satterlee, L.D. and Zachariah, N.Y. 1972. Porcine and ovine myoglobin: isolation, purification, characterization and stability. J. Food Sci. 37: 909-912.

Schricker, B.R., Miller, D.D. and Stouffer, J.R. 1982. Measurement and content of nonheme and total iron in muscle. J. Food Sci. 47: 740-743.

Sepe, H.A., Faustman, C., Lee, S., Tang, J., Suman, S.P. and Venkitanarayanan, K.S. 2005. Effects of reducing agents on premature browning in ground beef. Food Chem. 93: 571-576.

Setiowaty, G., Che Man, Y.B., Jinap, S. and Moh, M.H. 2000. Quantitative determination of peroxide value in thermally oxidized palm olein by Fourier transform infrared spectroscopy. Phytochem. Anal. 11: 74-78.

Shahidi, F. 1994. Assesment of lipid oxidation and off-flavour development in meat and meat products. *In* Flavor of Meat and Meat Products. (Shahidi, F., ed.). p. 247-266. Blackie Academic and Professional, London.

Shiraki, K., Kudou, M., Fujiwara, S., Imanaka, T. and Takagi, M. 2002. Biophysical effect of amino acids on the prevention of protein aggregation. J. Biochem. 132: 591-595.

Siddaiah, D., Sagar Reddy, G.V., Raju, C. and Chandrasekhar, T. 2001. Changes in lipids, proteins and kamaboko forming ability of silver carp (*Hypophthalmichthys molitrix*) mince during frozen storage. Food Res. Int. 34: 47-53.

Sivertsvik, M., Rosnes, J., Vorre, A., Randell, K., Ahvenainen, R. and Bergslien, H. 1999. Quality of whole gutted salmon in various bulk packages. J. Food Qual. 22: 387-401.

Sivertsvik, M., Jeksrud, W.K. and Rosnes, J.T. 2002. A review of modified atmosphere packaging of fish and fishery products – significance of microbial growth, activities and safety. Int. J. Food Sci. Technol. 37: 107-127.

Slabyi, B.M. and Hultin, H.O. 1982. Lipid peroxidation by microsomal fractions isolated from light and dark muscles of herring (*Clupea harengus*). J. Food Sci. 47: 1395-1398.

Smulevich, G., Droghetti, E., Focardi, C., Coletta, M., Ciaccio, C. and Nocentini, M. 2007. A rapid spectroscopic method to detect the fraudulent treatment of tuna fish with carbon monoxide. Food Chem. 101: 1071-1077.

Sohn, J.H., Taki, Y., Ushio, H., Kohata, T., Shioya, I. and Ohshima, T. 2005. Lipid oxidations in ordinary and dark muscles of fish: influences on rancid off-odor development and color darkening of yellowtail flesh during ice storage. J. Food Sci. 70: 490-496.

Sorheim, O., Aune, T. and Nesbakken, T. 1997. Technological, hygienic and toxicological aspects of carbon monoxide used in modified-atmosphere packaging of meat. Trends Food Sci. Technol. 8: 307-312.

Sorheim, O., Nissen, H. and Nesbakken, T. 1999. The storage life of beef and pork packaged in an atmosphere with low carbon monoxide and high carbon dioxide. Meat Sci. 52: 157-164.

Stadtman, E.R. 2001. Protein oxidation in aging and age-related diseases. Annals-New York Academy of Sciences 928: 22-38.

Steel, R.G.D. and Torrie, J.H. 1980. Principles and procedures of statistics; a biometrical approach. 2 nd Ed. McGraw-Hill Book. New York.

Stryer, L. 1988. Oxygen-transporting proteins: myoglobin and hemoglobin of lower vertebrates. Freeman. New York.

Suman, S.P., Mancini, R.A. and Faustman, C. 2006. Lipid-oxidation-induced carboxymyoglobin oxidation. J. Agric. Food Chem. 54: 9248-9253.

Surette, M.E., Gill, T.A. and LeBlanc, P.J. 1988. Biochemical basis of postmortem nucleotide catabolism in cod (*Gadus morhua*) and its relationship to spoilage. J. Agric. Food Chem. 36: 19-22.

Suzuki, T. and Imai, K. 1998. Evolution of myoglobin. Cell. Mol. Life Sci. 54: 979-1004.

Swatland, H.J. 1989. A review of meat spectrophotometry (300 to 800 nm). Can. Inst. Food Sci. Technol. J. 22: 390-402.

Tada, T., Watanabe, Y., Matsuoka, A., Ikeda-Saito, M., Imai, K., Ni-hei, Y. and Shikama, K. 1998. African elephant myoglobin with an unusual autoxidation behavior: comparison with the H64Q mutant of sperm whale myoglobin. Biochim. Biophys. Acta. 1387: 165-176.

Taguri, T., Tanaka, T. and Kouno, I. 2004. Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. Biol. Pharmaceut. Bull. 27: 1965-1969.

Tang, J., Faustman, C. and Hoagland, T.A. 2004. Krzywicki revisited: equations for spectrophotometric determination of myoglobin redox forms in aqueous meat extracts. J. Food Sci. 69: 717-720.

Tang, J., Faustman, C., Hoagland, T.A., Mancini, R.A., Seyfert, M. and Hunt, M.C. 2005a. Postmortem oxygen consumption by mitochondria and its effects on myoglobin form and stability. J. Agric. Food Chem. 53: 1223-1230.

Tang, J., Faustman, C., Mancini, R.A., Seyfert, M. and Hunt, M.C. 2005b. Mitochondrial reduction of metmyoglobin: dependence on the electron transport chain. J. Agric. Food Chem. 53: 5449-5455.

Tang, Q., Kalsbeck, W.A., Olson, J.S. and Bocian, D.F. 1998. Disruption of the heme iron-proximal histidine bond requires unfolding of deoxymyoglobin. Biochemistry. 37: 7047-7056.

Tang, S., Sheehan, D., Buckley, D.J., Morrissey, P.A. and Kerry, J.P. 2001. Anti-oxidant activity of added tea catechins on lipid oxidation of raw minced red meat, poultry and fish muscle. Int. J. Food Sci. Technol. 36: 685-692.

Thiansilakul, Y., Benjakul, S. and Richards, M. 2010. Changes in heme proteins and lipids associated with off-odour of seabass (*Lates calcarifer*) and red tilapia (*Oreochromis mossambicus* x *O. niloticus*) during iced storage. Food Chem. 121: 1109-1119.

Thiansilakul, Y., Benjakul, S. and Richards, M.P. 2011a. Effect of myoglobin from Eastern little tuna muscle on lipid oxidation of washed Asian seabass mince at different pH conditions. J. Food Sci. 76: C242-C249.

Thiansilakul, Y., Benjakul, S. and Richards, M.P. 2011b. Isolation, characterisation and stability of myoglobin from Eastern little tuna (*Euthynnus affinis*) dark muscle. Food Chem. 124: 254-261.

Thiansilakul, Y., Benjakul, S. and Richards, M.P. 2011c. The effect of different atmospheric conditions on the changes in myoglobin and colour of refrigerated Eastern little tuna (*Euthynnus affinis*) muscle. J. Sci. Food Agric. 91: 1103-1110.

Tichivangana, J. and Morrissey, P. 1985. Metmyoglobin and inorganic metals as pro-oxidants in raw and cooked muscle systems. Meat Sci. 15: 107-116.

Trout, G.R. and Gutzke, D.A. 1996. A simple, rapid preparative method for isolating and purifying oxymyoglobin. Meat Sci. 43: 1-13.

Uchida, K., Kato, Y. and Kawakishi, S. 1990. A novel mechanism for oxidative cleavage of prolyl peptides induced by the hydroxyl radical. Biochem. Biophys. Res. Commun. 169: 265-271.

Ueki, N. and Ochiai, Y. 2004. Primary structure and thermostability of bigeye tuna myoglobin in relation to those of other scombridae fish. Fish. Sci. 70: 875-884.

Undeland, I., Hultin, H.O. and Richards, M.P. 2003. Aqueous extracts from some muscles inhibit hemoglobin-mediated oxidation of cod muscle membrane lipids. J. Agric. Food Chem. 51: 3111-3119.

Van Dael, H., Haezebrouck, P., Pardon, E. and Joniau, M. 1997. Conformational stability of LYLA1, a synthetic chimera of human lysozyme and bovine alpha-lactalbumin. Eur. Biophys. J. 25: 171-179.

Van de Voort, F.R., Ismail, A.A., Sedman, J., Dubois, J. and Nicodemo, T. 1994. The determination of peroxide value by fourier transform infrared spectroscopy. J. Am. Oil Chem. Soc. 71: 921-926.

Van Der Zee, J., Barr, D.P. and Mason, R.P. 1996. ESR spin trapping investigation of radical formation from the reaction between hematin and tert-butyl hydroperoxide. Free Radical Biol. Med. 20: 199-206.

Vareltzis, K., Koufidis, D., Gavriilidou, E., Papavergou, E. and Vasiliadou, S. 1997. Effectiveness of a natural Rosemary (*Rosmarinus officinalis*) extract on the stability of filleted and minced fish during frozen storage. Eur. Food Res. Technol. 205: 93-96.

Vareltzis, P., Hultin, H.O. and Autio, W.R. 2008. Hemoglobin-mediated lipid oxidation of protein isolates obtained from cod and haddock white muscle as affected by citric acid, calcium chloride and pH. Food Chem. 108: 64-74.

Varlet, V., Knockaert, C., Prost, C. and Serot, T. 2006. Comparison of odor-active volatile compounds of fresh and smoked salmon. J. Agric. Food Chem. 54: 3391-3401.

Vuletich, J.L., Osawa, Y. and Aviram, M. 2000. Enhanced lipid oxidation by oxidatively modified myoglobin: role of protein-bound heme. Biochem. Biophys. Res. Commun. 269: 647-651.

Wallace, W.J., Houtchens, R.A., Maxwell, J.C. and Caughey, W.S. 1982. Mechanism of autooxidation for hemoglobins and myoglobins. Promotion of superoxide production by protons and anions. J. Biol. Chem. 257: 4966-4977.

Wang, T., Jónsdóttir, R., Kristinsson, H.G., Thorkelsson, G., Jacobsen, C., Hamaguchi, P.Y. and Ólafsdóttir, G. 2010. Inhibition of haemoglobin-mediated lipid oxidation in washed cod muscle and cod protein isolates by *Fucus vesiculosus* extract and fractions. Food Chem. 123: 321-330.

Wazawa, T., Matsuoka, A., Tajima, G., Sugawara, Y., Nakamura, K. and Shikama, K. 1992. Hydrogen peroxide plays a key role in the oxidation reaction of myoglobin by molecular oxygen. A computer simulation. Biophys. J. 63: 544-550.

Weiss, S.J. 1982. Neutrophil-mediated methemoglobin formation in the erythrocyte. The role of superoxide and hydrogen peroxide. J. Biol. Chem. 257: 2947.

Wilkinson, B.H.P., Janz, J.A.M., Morel, P.C.H., Purchas, R.W. and Hendriks, W.H. 2006. The effect of modified atmosphere packaging with carbon monoxide on the storage quality of master-packaged fresh pork. Meat Sci. 73: 605-610.

Wittenberg, B.A. and Wittenberg, J.B. 1987. Myoglobin-mediated oxygen Delivery to mitochondria of isolated cardiac myocytes. Proceedings of the National Academy of Sciences. 84: 7503-7507.

Yamaguchi, K., Takeda, N., Ogawa, K. and Hashimoto, K. 1979. Properties of mackerel and sardine myoglobins. Bull. Jap. Soc. Sci. Fish. 45: 1335-1339.

Yin, M.C. and Faustman, C. 1993. Influence of temperature, pH, and phospholipid composition upon the stability of myoglobin and phospholipid: A liposome model. J. Agric. Food Chem. 41: 853-857.

Zhu, H., Liu, M. and Lei, B. 2008. The surface protein Shr of *Streptococcus pyogenes* binds heme and transfers it to the streptococcal heme-binding protein Shp. BMC Microbiol. 8: 15.

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List of Publications and Proceedings

Publications

1. Thiansilakul, Y., Benjakul, S. and Richards, M. 2010. Changes in heme proteins and lipids associated with off-odour of seabass (*Lates calcarifer*) and red tilapia (*Oreochromis mossambicus* × *O. niloticus*) during iced storage. Food Chem. 121: 1109-1119.
2. Thiansilakul, Y., Benjakul, S. and Richards, M.P. 2011. Isolation, characterisation and stability of myoglobin from Eastern little tuna (*Euthynnus affinis*) dark muscle. Food Chem. 124: 254-261.
3. Thiansilakul, Y., Benjakul, S. and Richards, M. 2011. The effect of Fenton's reactants and aldehydes on the changes of myoglobin from Eastern little tuna (*Euthynnus affinis*) dark muscle. Eur. Food Res. Technol. 232: 221-230.

4. Thiansilakul, Y., Benjakul, S. and Richards, M.P. 2011. The effect of different atmospheric conditions on the changes in myoglobin and colour of refrigerated Eastern little tuna (*Euthynnus affinis*) muscle. J. Sci. Food Agric. 91: 1103-1110.
5. Thiansilakul, Y., Benjakul, S. and Richards, M.P. 2011. Effect of myoglobin from Eastern little tuna muscle on lipid oxidation of washed Asian seabass mince at different pH conditions. J. Food Sci. 76: C242-C249.
6. Thiansilakul, Y., Benjakul, S., Sung Yong, P. and Richards, M. 2011. Characteristics of myoglobin and hemoglobin-mediated lipid oxidation in washed mince from bighead carp (*Hypophthalmichthys nobilis*). Submitted.
7. Thiansilakul, Y., Benjakul, S. and Richards, M. 2011. Retardation of myoglobin and hemoglobin-mediated lipid oxidation in washed bighead carp by phenolic compounds. Submitted.
8. Thiansilakul, Y., Benjakul, S. and Richards, M.P. 2011. Effect of phenolic compounds in combination with modified atmospheric packaging on retardation of quality losses of refrigerated Eastern little tuna slices. Submitted.

Proceedings

1. Thiansilakul, Y., Benjakul, S. and Richards, M.P. 2010. Effect of Fenton's reactants and aldehydes on the changes of myoglobin from Eastern little tuna (*Euthynnus affinis*) dark muscle. The 55th Annual Fisheries Technology Conference. St.John's, Newfoundland, Canada. September 7-10, 2010. Poster presentation.
2. Thiansilakul, Y., Benjakul, S. and Richards, M.P. 2011. Characteristic of pro-oxidative activity of myoglobin and hemoglobin in washed Asian carp (*Hypophthalmichthys nobilis*) stored in ice. The 62nd Pacific Fisheries Technologists Conference. Vancouver, British Columbia, Canada. February 13-16, 2011. Poster presentation.
3. Thiansilakul, Y., Benjakul, S. and Richards, M.P. 2011. Comparative study on pro-oxidative activity of myoglobin and hemoglobin in washed Asian carp (*Hypophthalmichthys nobilis*) mince during iced storage. RGJ Seminar Series LXXXIII: Natural Resources and Management for Sustainable Utilization. Prince of Songkla University, Hat Yai, Songkhla. August 31, 2011. Oral presentation.