Studies on the myoglobin reduction mechanism in red-fleshed fish meat

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mechanism in red-fleshed fish meat

（赤身魚肉のミオグロビン還元機構に関する研究）

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Contents

Background

As an indicator of the freshness and quality, meat color can influence consumers acceptability and purchase decisions (Al-Shaibani et al. 1977b). Meat color greatly depends on the content and state of muscle pigment, most of which is myoglobin (Mb). Mb is an oxygen-binding heme protein, responsible for intracellular oxygen storage, buffering intracellular oxygen concentrations and facilitation of oxygen diffusion. Depending on its redox state, Mb exists as either of three derivatives, namely, deoxymyoglobin (deoxyMb), oxymyoglobin (oxyMb) and metmyoglobin (metMb). DeoxyMb contains ferrous iron at the center of heme and thus gives dark purplish red color. OxyMb is formed by oxygenation of deoxyMb, which has a diatomic oxygen attached to the sixth coordination site of the ferrous iron. Therefore, saturating Mb with oxygen provides attractive bright-red color to meat because of oxyMb formation. The oxidation of the ferrous form to a ferric state results in the formation of brown metMb. MetMb has a water molecule bound at the sixth coordination site of the ferric iron, which is incapable of binding oxygen. The formation and accumulation of metMb are associated with meat discoloration (AMSA 2012). Generally, in living muscles, metMb is not accumulated, as metMb-reducing enzyme systems maintain the physiological role of Mb and could be relevant to the maintenance of fresh meat color. In this connection, the presence of NADH-cytochrome b_5 reductase and an electron carrier (cytochrome b_5 located in the outer membrane of mitochondria) has been verified in the mitochondrial fraction of bovine muscle (Arihara et al. 1995). Furthermore,

mitochondria have an ability to keep color stability of beef via mitochondria-mediated metMb reducing activity. Although the researches on the role of mitochondria in beef color and its stability have been frequently discussed, very little information is available on mitochondrial function in fish muscle in relation with metMb reduction and meat color stability. Many migratory fish species, such as tuna, have considerable amount of Mb, and consequently, undesirable fast meat discoloration is encountered when improperly stored.

Therefore, the objective of this study was to explore the role of mitochondrial fraction in metMb reduction in tuna meat.

1. Meat color

For fresh meat, color is an important sensory property that greatly influences consumers' perceptions of quality (Ramanathan et al. 2020). Color is one of the most important indices for consumers to evaluate the freshness and quality of raw meat, as consumers believe that color is related to microbial load and/or safety indicators. Nevertheless, the color of fresh meat is not a good predictor of total bacterial load (Chan et al. 1996). Although meat color is not a reliable predictor for its safety and quality, it does not prevent consumers from having certain and specific expectations (Tomasevic et al. 2021). It is often used as an indicator of meat quality and wholesomeness on which they base their purchasing decisions. In a meat quality survey in Japan, 58% of all participants (n=10,941) identified meat color as the most important

factor in selecting beef products (Sanders et al. 1997). The economic consequences resulting from consumer discrimination against meat color and the resulting price discounts and product discards become significant (Ramanathan et al. 2020). Discarded discolored meat not only has direct economic impacts, but also cause environmental waste. Therefore, developing future approaches to improve color stability is critical for avoiding adverse economic and potential environmental consequences resulting from discolored meats.

The present study focused on red-fleshed fish meat. The color of fish and fishery products is also used as a consumers' indicator of freshness, which affects their acceptability and purchase decisions (Faustman and Cassens 1990). Matthews (1983) found that storage life on ice before noticeable browning occurred was 12-14 days for yellowfin tuna and 7 days for bonito and skipjack tuna. Previous researches suggested the formation of metMb with increasing storage time is the main cause in the development of the unpleasant color in fish muscles (Chiou et al. 2001; Chaijan et al. 2005; Sohn et al. 2005).

2. Mb

Meat color depends on the content and state of muscle pigment, most of which is Mb. Mb is an intracellular globular heme protein found in skeletal and cardiac muscles of vertebrates (Giddings 1977; Livingston et al. 1983). The chemistry and functions of Mb in living muscles and meats can be different (AMSA 2012). In living muscles, Mb

functions as the oxygen binder and delivers oxygen to the mitochondria, and enables the tissue to maintain its physiological functions (Wittenberg and Wittenberg 1989). However, in meats, Mb serves as the major pigment responsible for red color of meats.

A prosthetic heme group containing an iron atom at the center is positioned in the hydrophobic core of Mb protein (globin). Of the six bonds related to this iron atom, four are connected to the heme ring, the fifth site attaches to the proximal histidine-93 (mammalian Mb), and the sixth site is available to reversibly bind ligands, such as diatomic oxygen and water. The ligand present at the sixth coordination site and the valence state of iron determine meat color via three chemical forms of Mb, deoxyMb, oxyMb and metMb, and the relative proportions of these three derivatives determine the color of fresh meat. (Stryer 1995; Bekhit and Faustman 2005; Mancini and Hunt 2005; AMSA 2012).

DeoxyMb contains ferrous iron with a vacant (without any ligand attached) sixth coordination site, results in the dark purplish red color. When Mb is exposed to oxygen, oxyMb generates through Mb oxygenation which is characterized by the development of a bright cherry-red color. While the sixth coordination site is occupied by diatomic oxygen, there is no change in iron's valence during Mb oxygenation. Moreover, the distal histidine interacts with bound oxygen to alter the structure and stability of Mb (Mancini and Hunt 2005). Undergoing spontaneous oxidation of Mb, ferrous Mb is oxidized to the ferric state to form metMb, which is undesirable brown in color and cannot bind oxygen (Bekhit and Faustman 2005). MetMb has a water molecule bound at the sixth coordination site of the ferric heme, thereby preventing the oxygen binding.

In fresh cut meat, because deoxyMb occupies the majority due to lack of oxygen, the surface is usually purplish red. After being exposed to the air for several minutes at room temperature, the meat surface will become bright red because of the formation of oxyMb. MetMb formation tends to initiate beneath the surface between the superficial oxyMb and interior deoxyMb, where oxygen partial pressure is not high enough to oxygenate all available deoxyMb. Thus, some deoxyMb is available to react with oxygen radicals to form metMb.

All three forms of Mb are easily soluble in water and low-ionic strength buffers, and the visible absorbance spectra of the redox forms are different enough to be identified spectrophotometrically (Krzywicki 1982; Tang et al. 2004; AMSA 2012). Tang and co-workers (2004) reported deoxyMb and metMb exhibit a strong absorption maximum at 557 and 503 nm, respectively, and oxyMb has two large peaks at 542 and 582 nm. The absorption spectra of the three derivatives intersect at 525 nm (isobestic point), and the spectrophotometric absorbance at 525 nm was used to estimate total Mb concentration in solutions as well as in the fresh meat extracts.

3. MetMb reduction

Oxidation of ferrous Mb to ferric metMb, and subsequent accumulation of metMb results in the discoloration (browning) of meat color. In addition to species-dependent effects, variation in meat color also depends on many internal factors (gender, breed, endogenous antioxidants, age, muscle type and metabolism, the ultimate pH of the

meat, and the rate of post-mortem pH decline) and external factors (temperature, oxygen availability, type of light exposed, packaging, type and growth of surface microorganisms) (Bekhit and Faustman 2005).

Generally, there is no appreciable accumulation of metMb *in vivo*, as metMb-reducing enzyme systems maintain the physiological role of Mb and could be relevant to the maintenance of fresh muscle color (Rossi-Fanelli et al. 1957; Giddings and Hultin 1974; Giddings 1977; Hagler et al. 1979; Echevarne et al. 1990). Dean and Ball (1960) reported the existence of natural metMb reducing systems in beef. NADH-cytochrome b_5 reductase responsible for methemoglobin (metHb) and metMb reduction was identified in erythrocyte, which is a flavoprotein (flavin-containing dehydrogenase) and is bound tightly to endoplasmic reticulum membranes (microsomes) or the outer mitochondrial membrane (Strittmatter and Velick 1956; Takesue and Omura 1970a, 1970b; Shirabe et al. 1992). Not surprisingly, immunological similarities between metHb and metMb reductases have been reported (Kuma et al. 1976). Livingston et al. (1985) provided evidence for the mechanism by which NADH-cytochrome b_5 reductase utilizes NADH to reduce ferricytochrome b_5 to ferrocytochrome b_5. Then, ferrocytochrome b_5 reduces metMb to ferrous Mb non-enzymatically. Meanwhile, it was also reported metMb and the cofactor cytochrome b_5 bind to the enzyme in a one-to-one stoichiometry *in vitro*. Subsequently, Shirabe et al. (1992) also demonstrated that the function of NADH-cytochrome b_5 reductase is to transfer two electrons from NADH to two molecules of cytochrome b_5, and reduced cytochrome b_5 then transfers these electrons to a variety of acceptors including metHb

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or metMb. Bailey and Driedzic (1992) supported the argument that this enzyme is responsible for reducing metMb *in vivo*. The presence of NADH-cytochrome b_5 reductase and an electron carrier (cytochrome b_5 located in the outer membrane of mitochondria) in the mitochondrial fraction of bovine muscle was verified (Arihara et al. 1995). Furthermore, mitochondria have an ability to affect color stability of beef via mitochondria-mediated metMb reducing activity (Ledward 1985; English et al. 2016; Ramanathan et al. 2010).

Although the mitochondrial researchers related to beef color and its stability were frequently discussed (Tang et al. 2005a, 2005b; Ramanathan et al. 2010; Ramanathan and Mancini 2010; Mancini and Ramanathan 2014; Belskie et al. 2015), no previous research has examined the role of mitochondrial function in fish muscle in relation with metMb reduction and color stability. Therefore, the objective of this study was to explore the relationship of mitochondrial fraction and metMb reduction in the tuna meat.

Chapter 1: The presence of NADH-dependent metMb reductase in the mitochondrial fraction

1. Background

Cytochrome b_5 reductase was first purified by solubilization from sub-cellular membranes by incubation of liver microsomes with cobra venom (Strittmatter and Velick 1956; 1957). The proteases in the venom were the agent responsible for solubilization of the 33 kDa protein (Takesue and Omura 1970a; 1970b). This protease-solubilized form of the flavoprotein is a hydrophilic fragment of the intact molecule produced by proteolytic removal of the hydrophobic segment that anchors the enzyme in membranes (Takesue and Omura 1970a; 1970b).

Subsequently, a detergent-solubilized form of flavoprotein was prepared from the membranes (Spatz and Strittmatter 1973; Mihara and Sato 1975). This produces the amphiphilic reductase in its native form. It consists of a hydrophilic moiety, which contains the active sites FAD (the reductase) and heme (cytochrome b_5) projecting into the surrounding cytoplasm (Williams 1976). The other component is the hydrophobic peptide segment responsible for binding the flavoprotein to the microsomal membranes. The protein solubilized by detergent has a molecular weight of 44 kDa and aggregates in an aqueous medium (Spatz and Strittmatter, 1973). This form is different from the venom protease-derived form in that it can bind to a variety of membrane preparations (such as liver microsomes, liver mitochondria, and egg yolk phosphatidylcholine) *in vitro*, while

protease-soluble flavoprotein cannot (Mihara and Sato, 1978). The activity of this cytochrome b_5 reductase was determined by measuring its ability to reduce ferricyanide utilizing NADH (Mihara and Sato, 1975; 1978). Williams (1976) suggested that the turnover numbers of the detergent-extracted reductase in the reduction of ferricyanide or detergent-extracted cytochrome b_5 by NADH are 21% and 77% lower than that of the soluble proteins, respectively. Moreover, Spatz and Strittmatter (1973) found that the reduction of ferricyanide catalyzed by the reductase was nearly normal, but the reduction of cytochrome b_5 was inhibited by the reductase aggregation in the presence of detergent. NADH-cytochrome b_5 reductase is essentially specific for NADH, as NADPH-dependent reduction rate was only 0.001% of that in the presence of NADH (Bekhit and Faustman 2005).

NADH-cytochrome b_5 reductase is not only classified according to its solubilized forms, but a new classification system based on location is adopted. Cytochrome b_5 reductase can be prepared in soluble and membrane-bound forms. The soluble form exists in erythrocytes and participates in the metHb reduction, while the membrane-bound form is located on the endoplasmic reticulum and on the outer mitochondrial membranes which involves metMb reduction and plays a role on the elongation and desaturation of fatty acids, cholesterol biosynthesis, and cytochrome P-450-mediated drug metabolism (Shirabe et al., 1992). MetMb reductase is different from metHb reductase based on the difference in isoelectric point and specific activity (Shimizu and Matsuura 1968; 1971). However, the purified enzyme is not specific for metMb, while both metHb and metMb reductases displayed higher activities with metMb and metHb, respectively (Shimizu and

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Matsuura 1968; 1971).

Hagler et al. (1979) reported an apparent specific metMb reducing enzyme. The NADH-dependent metMb reductase from bovine heart required ferrocyanide and NADH for metMb reducing activity. They also demonstrated that erythrocyte and bovine heart reductase could reduce metMb and metHb at different rates. Similarly, Yubisui and Takeshita (1980) and Tamura et al. (1983) purified NADH-cytochrome b_5 reductase from human red blood cells, bovine brain, bovine liver microsomes and rabbit red blood cells, respectively. Güray and Arinç (1990, 1991) and Yang and Cederbaum (1994) purified NADH-cytochrome b_5 reductase from sheep lung, rat and rabbit liver microsomes. Arihara et al. (1995) localized NADH-cytochrome b_5 reductase to the mitochondrial fraction of bovine skeletal muscle and at a lower level in the microsomal fraction. In summary, NADH-cytochrome b5 reductase has been identified in a variety of tissues.

In addition to the researches on mammals, there are few studies on fish muscles. MetMb reductase from bluefin tuna (*Thunnus thynnus*) was purified by Sephadex G-25 gel filtration, ammonium sulfate fractionation and Sephadex G-71 gel filtration. Similarly, metMb reductase from bluefin tuna ordinary muscle was purified to electrophoretical homogeneity by ammonium sulfate fractionation, ion exchange and organomercurial agarose affinity chromatography (Pong et al. 2000b). Pong et al. (2000a) also used NADPH and methylene blue to study the effect of polyethylene packaging on metMb reductase activity and the color of tuna muscle during storage at 4 ℃. Chiou et al. (2001) determined the *in vitro* reducing ability of metMb reductase. Briefly, in which discolored bluefin tuna muscle was immersed into metMb reductase solution (0.103 unit/ml) for 120

min at 4 ℃. Therefore, the purpose of this chapter was to prepare a mitochondrial fraction from the dark muscle of tuna and demonstrate the presence of metMb reductase.

2. Materials and methods

2.1. Materials

Fresh specimens of the dark muscle of bigeye tuna (*Thunnus obesus*) was purchased at Shiogama Seafood Market, Miyagi Prefecture, Japan, on May 25th, 2019, and stored at -80 ℃ until use.

2.2. Chemicals

Mb from equine skeletal muscle (95-100%) and NADH ($\geqq$97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium hexacyanoferrate (Ⅱ) trihydrate was obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). EDTA Na$_2$ was from Dojindo (Kumamoto, Japan). Tris (hydroxymethyl) aminomethane, hydrochloric acid, sucrose, (±) dithiothreitol (DTT) and 0.1 mol/l phosphate buffer solution (pH 6.0, 6.4 and 7.0) were obtained from Fuji Film Wako Pure Chemical (Osaka, Japan). All the chemicals used were of analytical grade. 4, 6-diamidino-2-phenylindole (DAPI) solution (10 mg/ml) was from PromoCell (Heidelberg, Germany).

2.3. Preparation of mitochondrial fraction - Ultra-centrifugation method

Mitochondrial fractions were isolated from tuna dark muscle according to Boocock

et al. (1970) and Suzuki et al. (1981). The dark muscle was homogenized with 5 volumes of iced-cold grinding solution (0.5 M sucrose; 10 mM EDTA Na$_2$; 1 mM DTT; 50 mM Tris-HCl buffer, pH 7.5) using a grinder (Coyote Tissue Grinder G50) at 3,000 rpm for 1 min on ice, and centrifuged at 1,000×g for 10 min. The supernatant was further centrifuged at 10,000×g for 20 min to precipitate the mitochondria. The sediment was dissolved with 5 volumes of wash solution (0.5 M sucrose; 1 mM DTT; 20 mM Tris-HCl buffer, pH 7.5) and precipitated again at 10,000×g. The crude mitochondria fraction thus obtained was dissolved in 40% sucrose, and used for the following experiments.

Aliquots of 3 ml of 40% and 60% sucrose were placed into 6.5 ml centrifuge seal tubes and stratified depending on the difference in specific gravity of the solutions. The mitochondria were precipitated at the boundary of the solutions by centrifugation at 120,000×g for 1 h, and subsequently subtracted with a 1 ml syringe equipped with a needle (0.60φ×25 mm). The mitochondria fraction was suspended in 1 ml of 5% sucrose and centrifuged at 10,000×g for 30 min centrifugation. The sediment was re-dissolved in 200 μl of the wash solution and stored at 4 ℃ until use (for up to one week). All the extract steps were performed at 0-4 °C.

2.4. MetMb reduction

The reduction activity of metMb was measured according to Halger et al. (1979) in a 1 ml cuvette which contained the mixture (final pH 4.9) of 0.50 μmol EDTA Na$_2$, 5.0 μmol pH 4.7 citrate buffer, 0.30 μmol K$_4$Fe(CN)$_6$, 0.15 μmol metMb in 20 mM pH 6.0

sodium phosphate buffer, 50 μl mitochondrial fraction, 10 μmol NADH, and a total volume was adjusted to 1.0 ml with distilled water. The absorption spectra were measured under 25 ℃ incubation, and the ratios of deoxyMb, oxyMb, and metMb were calculated by the following formulae (Tang et al. 2004):

$$[deoxyMb] = -0.543A_{582}/A_{525}+1.594A_{557}/A_{525}+0.552A_{503}/A_{525}-1.329$$

$$[oxyMb] = 0.722A_{582}/A_{525}-1.432A_{557}/A_{525}-1.659A_{503}/A_{525}+2.599$$

$$[metMb] = -0.159A_{582}/A_{525}-0.085A_{557}/A_{525}+1.262A_{503}/A_{525}-0.520$$

The control absorption spectrum was measured in the absence of NADH, and metMb reduction was initiated by the addition of NADH. One unit of metMb reductase activity was defined as one micromole of reduced metMb per min per gram of mitochondria protein.

3. Results and discussion

The mitochondrial fraction was prepared from the dark muscle of bigeye tuna (*Thunnus obesus*) by use of the density-gradient ultra-centrifugation method. The light microscopy mitochondrial fraction stained with DAPI is shown in Fig. 1, and similar to the DAPI staining of mitochondrial DNA reported by Zoladek et al. (1995). The edges of the mitochondria were vague, they may have been damaged and fragmented. Tang et al. (2005b) speculated the appearance of fragments in the samples of isolated mitochondria after 96 h and 60 days may have resulted from increased fragility and susceptibility to damage caused by isolation procedures.
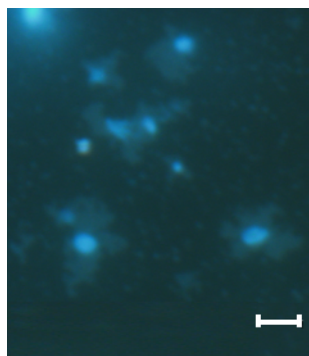
Fig. 1. DAPI staining of mitochondrial fraction extracted from bigeye tuna; Scale bar = 2 μm.

The metMb reducing activity was measured by monitoring the visible absorption spectra during incubation at 25°C in a 1 ml cuvette which contained the horse metMb, mitochondrial fraction, and an electron donor NADH. After 10 min incubation, the proportion of oxyMb increased by 8.1% and that of metMb decreased by 8.8%. However, this reduction reaction was completed within about 3 min (Fig. 2). The mitochondrial fraction and NADH were removed from the metMb reducing substrates respectively, and then the derivatives ratio of horse Mb was monitored (Fig. 3). As the results, there was no obvious metMb reducing activity without the mitochondrial fraction or NADH. Therefore, early termination of the metMb reduction was thought to be due to the consumption of an electron donor NADH.

Purified bovine metMb and ovine metMb, as described by Livingston et al. (1985), were reduced non-enzymatically by NADH (Bekhit et al., 2001). That non-enzymatic reduction of metMb is probably similar to that reported by Brown and Snyder (1969).

EDTA was found to be essential for non-enzymatic reduction of metMb by NADH (Brown and Snyder, 1969). Furthermore, the non-enzymatic reducing activity of NADH when ovine metMb was used in the assay accounted for about 50% of the total reducing activity, but non-enzymatic reduction was not observed when commercially available equine metmyoglobin was used as a substrate. (Bekhit et al., 2001). It might explain why no non-enzymatic reducing activity was observed in this experiment, because commercial equine metMb was used.

Livingston et al. (1985) found that the reduction rates of metMb increased more than 3-4 fold in the presence of ferrocyanide compared to cytochrome b_5 in a kinetic study of metMb reductase activity. Moreover, the presence of potassium ferrocyanide has been demonstrated to be essential for the reduction of metMb by metMb reductase (Hagler et al. 1979; Reddy and Carpenter 1991; Madhavi and Carpenter 1993; Mikkelsen et al. 1999) and erythrocyte metHb reductase (Hegesh and Avron 1967). Although cytochrome b_5 acts as the true electron transfer mediator, ferrocyanide forms a stoichiometric complex with the heme protein, which can enhance the rate of electron transfer (Livingston et al., 1985).

Equine metMb was clearly reduced by the mitochondrial fraction from the dark muscle of bigeye tuna as detected by the spectrophotometric assay. The rate of metMb reduction was calculated by Tang's formulae (Tang et al. 2004). The results supported the presence of a metMb reductase system in the mitochondrial fraction from the dark muscle of tuna. NADH was essential for metMb reduction as metMb was not reduced at all in its absence. It was in good agreement with the finding that equine and porcine

metMbs were enzymatically reduced by the porcine *longissimus dorsi* muscle extract
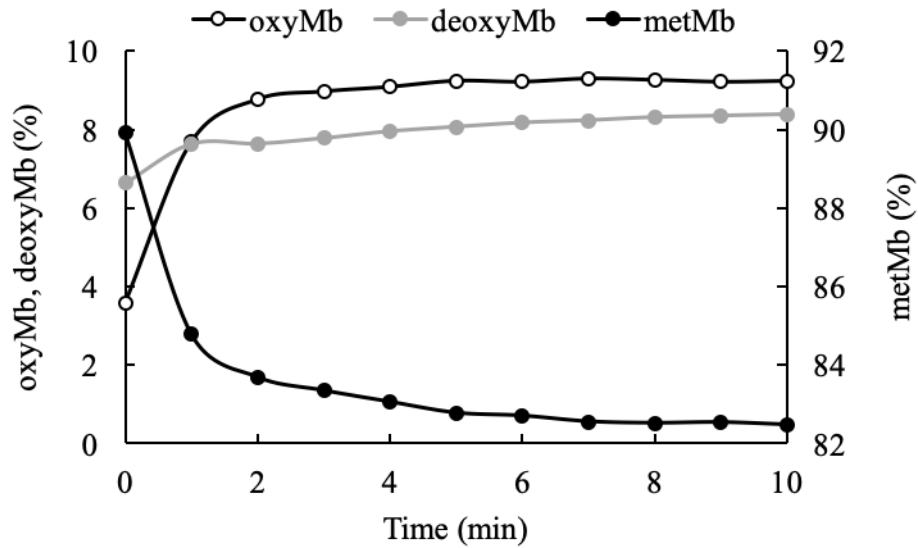
(Mikkelsen et al. 1999).

Fig. 2. Changes in the derivatives ratio of horse Mb in the metMb reducing substrates.
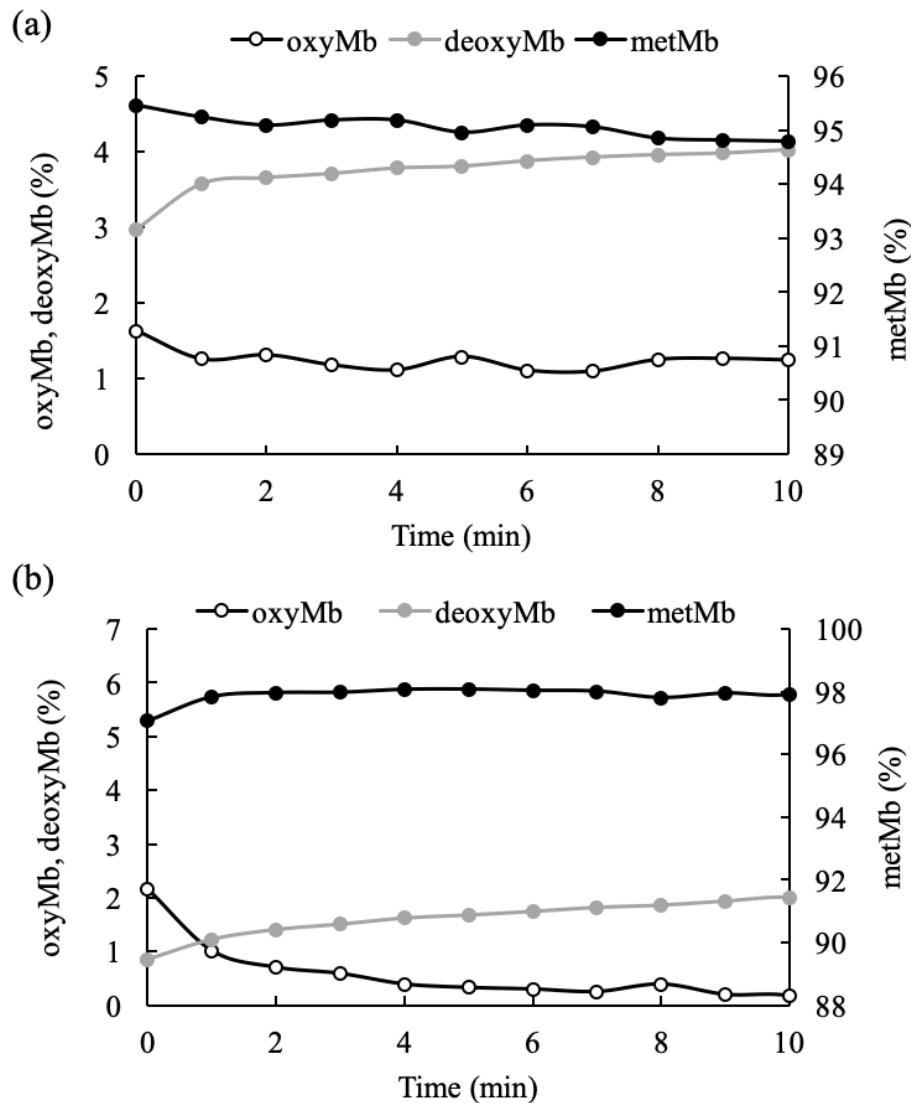
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Fig. 3. Changes in the derivatives ratio of horse Mb when the mitochondrial fraction (a) or NADH (b) was removed from the metMb reducing substrates.

In summary, the proportion of metMb decreased and that of oxyMb increased, suggesting the presence of NADH-dependent metMb reductase in the mitochondrial fraction. Since the bigeye tuna muscle used in this experiment has been stored frozen, it is possible that the activity of the metMb reductase was slightly reduced. Also, the use of crude extracts as a source of metMb reductase may not be entirely correct as these

fractions may contain other enzymes which are capable of reducing cytochrome b_5 (such

as the case with cytochrome p450-reductase) (Bekhit and Faustman 2005).

Chapter 2: Effects of temperature, pH and NADH concentration on the metMb reducing activity of the mitochondrial fraction

1. Background

According to Giddings and Hultin (1974), the loss of metMb reducing activity in post-rigor meat was attributed to the factors such as decrease in pH, depletion of substrates such as NADH, oxidative deteriorative changes, decreasing enzymatic activities including decomposition of mitochondrial particles. MetMb reduction is accelerated with increased temperature *in situ* (Stewart et al. 1965; Hutchins et al. 1967; Zimmerman and Snyder 1969). Stewart et al. (1965) found that the effect of temperature on metMb reducing activity depended on meat form (intact or minced). Similarly, optimum pH for metMb reducing activity seems to be dependent on the reducing activity source (purified preparation or crude extract) and assay conditions (Bekhit and Faustman 2005). In the case of tuna meat, the color stability is much lower compared with that of livestock meat, and thus discoloration proceeds much faster. The activation of metMb reduction system, if any, would greatly help the maintenance of meat color during storage.

Therefore, the objective of this chapter was to explore the relationship of mitochondrial fraction and metMb reduction in the dark muscle of tuna. Factors, such as assay temperature, pH, and cofactors like NADH, that could influence the mitochondrial functions related to the appearance of tuna dark muscle were also investigated.

2. Materials and methods

2.1. Materials

Fresh specimens of the dark muscle of bigeye tuna (*Thunnus obesus*) was purchased at Shiogama Seafood Market, Miyagi Prefecture, Japan, on Jun 20th, 2020, and stored at -80 ℃ until use.

2.2. Chemicals

Pierce BCA protein assay kit was from Thermo Fisher Scientific (Fair Lawn, New Jersey, USA). Other chemicals were the same as in Chapter 1.

2.3. Preparation of mitochondrial fraction - Ultra-centrifugation method

Preparation of mitochondrial fraction was the same as in Chapter 1.

2.4. Protein concentration determination

Protein concentration of the mitochondrial fraction was determined according to the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific) using bovine serum albumin as a standard. The protein concentration thus determined was used for calculation of metMb reductase activity, which was defined as the amount of metMb (μmol) reduced by gram of protein per min.

2.5. MetMb reduction

The reduction activity determination of metMb was similar to that in Chapter 1 according to Halger et al. (1979).

2.6. Statistical analysis

Statistical analysis was performed using Microsoft Excel 2010 (Statcel 4). The significant differences ($p < 0.05$) were determined by using ANOVA.

3. Results and discussion

In the process of measuring metMb reduction activity, oxygen in the air can affect metaMb reduction. Therefore, liquid paraffin was used to block the surface of the reaction solution to prevent the ingress of oxygen. As a result, there was no significant difference in the metMb reducing activity with or without liquid paraffin (Table 1, $p > 0.05$, t-test), indicating that oxygen in the air does not affect the measurement of the metMb reducing ability. Sammel et al. (2002) speculated aerobic reducing ability correlated best with color stability over display and appeared to be the best current method for measuring reducing ability.

Table 1. MetMb reductase activity of the mitochondrial fraction with or without liquid paraffin.

Incubation condition	MetMb reductase activity (μmol/min/g protein)
Control	85.9±5.9
Liquid paraffin	85.7±6.5

MetMb reductase activity (μmol/min/g protein): metMb (μmol) is reduced by 1 g mitochondrial protein per minute; All data are shown as averages with S.D. (n=3); t-test (p >0.05).

Effect of temperature

MetMb reduction was accelerated with increased temperature (10-30℃) and showed the highest activity at 30℃ (Fig. 4). However, during the 10 min incubation, there was no significant different changes in relative metMb (%). Increase in temperature could promote the metMb reducing activity of mitochondrial fraction, but did not influence the metMb reducing capacity.

Optimum temperature for metMb reducing activity appears to be species- and reductase source (purified preparation vs. extract)-dependent (Bekhit and Faustman 2005). In this study, increased temperature resulted in acceleration of metMb reduction. Similarly, Reddy and Carpenter (1991) found that increased temperature from 4 to 30℃ tripled metMb reducing activity in the bovine *l. dorsi* muscle extract at pH 6.4 and 7.0. Mikkelsen et al. (1999) reported that the rates of enzymatic and non-enzymatic reduction of metMb in the porcine *l. dorsi* muscle increased in a temperature-dependent

manner in the range of 15-30℃. Regarding the activity from fish sources, purified yellowfin tuna (*Thunnus albacares*) metMb reductase was most active in the temperature range of 33-35℃ (Levy et al. 1985), while the purified metMb reductase from bluefin tuna (*T. thynnus*) was found to show the optimal temperature of 25℃, but was mostly inactivated above 30℃. Al-Shaibani et al. (1977b) also showed the optimum temperature of the purified metMb reductases from bluefin tuna and jack mackerel (*T. symmetricus*) was 25℃. Consistent with the finding of Stewart et al. (1965), the effect of temperature on metMb formation might not be the same for all the sources, since increasing temperature accelerates not only metMb reductase activity but also Mb autoxidation. In the present study, there was no significant difference in metMb reductase activity between at 25℃ and 30℃. Therefore, the assays in the following experiments were performed at 25℃.
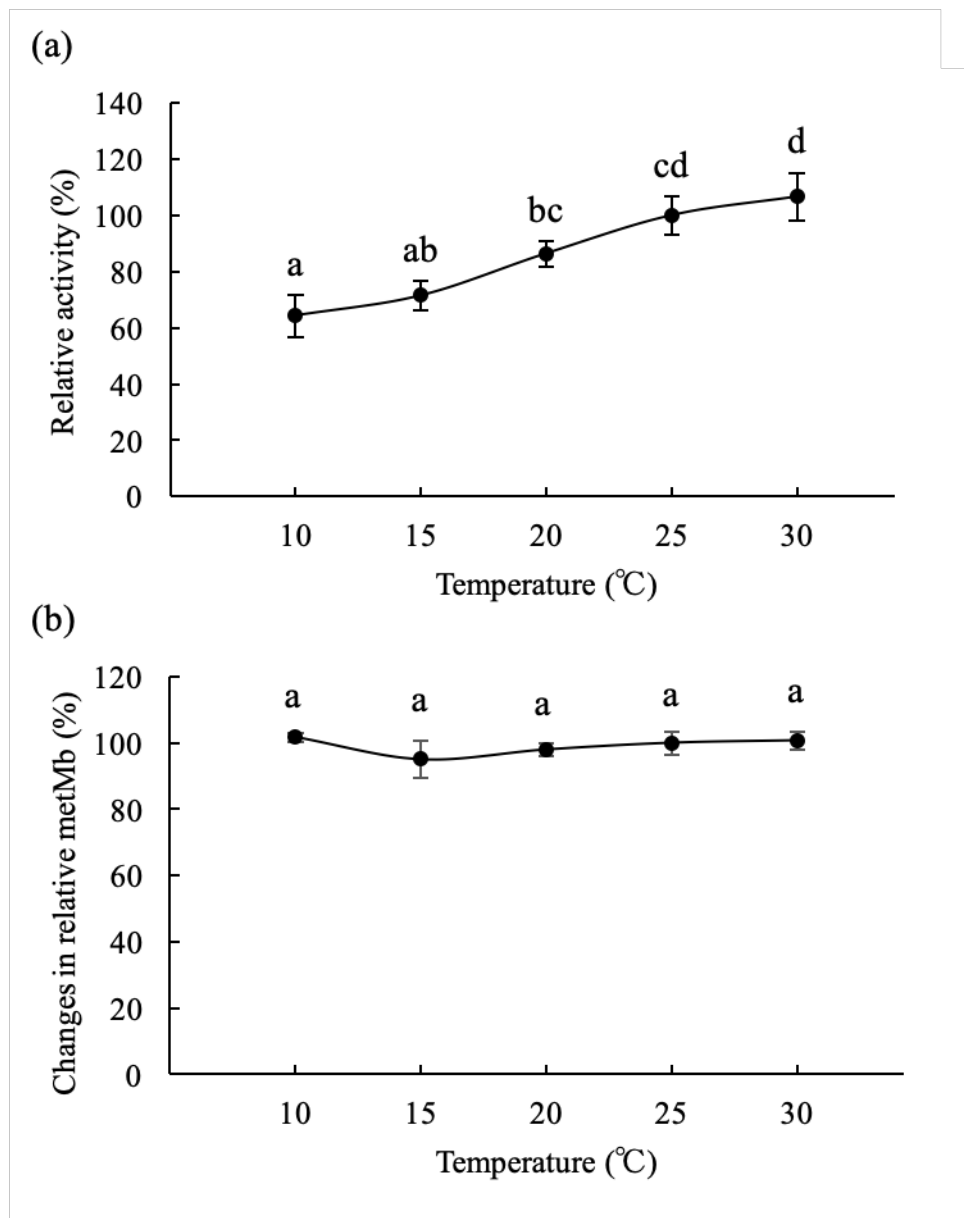
Fig. 4. Effect of temperature on reduction of the metMb. (a) relative activity (%): metMb reductase activity relative to the control (25 °C); metMb (μmol) was reduced by 1 g of mitochondrial protein per min. (b) changes in relative metMb (%): changes in metMb ratio against total Mb after 10 min reduction relative to the control (25 °C). All data are shown as averages with S.D. (n=3). Different superscripts in the same graph indicate significant differences ($p < 0.05$, Tukey-Kramer).

Effect of pH

MetMb reducing activity and changes in relative metMb (%) decreased with increased pH values (Fig. 5), but metMb reducing activities were shown to vary in the different buffers, even at the same pH. The optimal pH values of citrate buffer and phosphate buffer were 5.0 and 6.0, respectively (Fig. 5).

When metMb reduction was measured in the pH range of 5.7-7.0 using phosphate buffers, there was no significant difference in the activity from pH 5.7 to 6.8, but a significant decrease was found at pH 7.0 (Fig. 6). The maximal metMb reducing capacity was found at around pH 6.6-6.8 in the phosphate buffer after 10 min incubation.

In bovine cardiac muscle, a similar curve of metMb reductase activity related to assay pH was observed, when citrate buffer or acetate buffer was added to the assay mixture, and the optimal pH values in these two buffers were approximately 6.5 in the pH range of 5.7-7.3 (Hagler et al. 1979). In contrast, different optimal pH was observed in the citrate and phosphate buffers. Based on the postmortem pH change in muscle, the assay of pH effect on metMb reduction was investigated in the pH range of 5.7-7.0 using phosphate buffers. The results were consistent with those of porcine metMb reduction (Mikkelsen et al. 1999), showing that the metMb reductase activity decreased at higher pH. Nevertheless, maximal metMb reducing capacity (the amount of reduced metMb) was found at around pH 6.6-6.8 as suggested by the changes in the metMb ratio in the pH range of 5.7-7.0. Although metMb reductase activity of the lamb muscle was not affected in the pH range of 5.5-7.5, the numerically highest activity was

observed when the pH was 7.4 (Bekhit et al. 2001). Similarly, Echevarne et al. (1990) reported metMb reducing activity of bovine muscle homogenates was maximal at around pH 7.3, when phosphate buffers of varous pH were used. Al-Shaibani et al. (1977a) showed that metMb reductase activity from bluefin tuna (*Thunnus thynnus*) muscle was highest at around pH 7.0-7.3. It has been shown that optimum pH for metMb reductase activity is dependent on the enzyme source and assay conditions (Bekhit and Faustman 2005). It was thus considered that the enzyme in the mitochondrial fraction was stable against pH changes, as there was no obvious effect on its pH-dependency of metMb reduction.
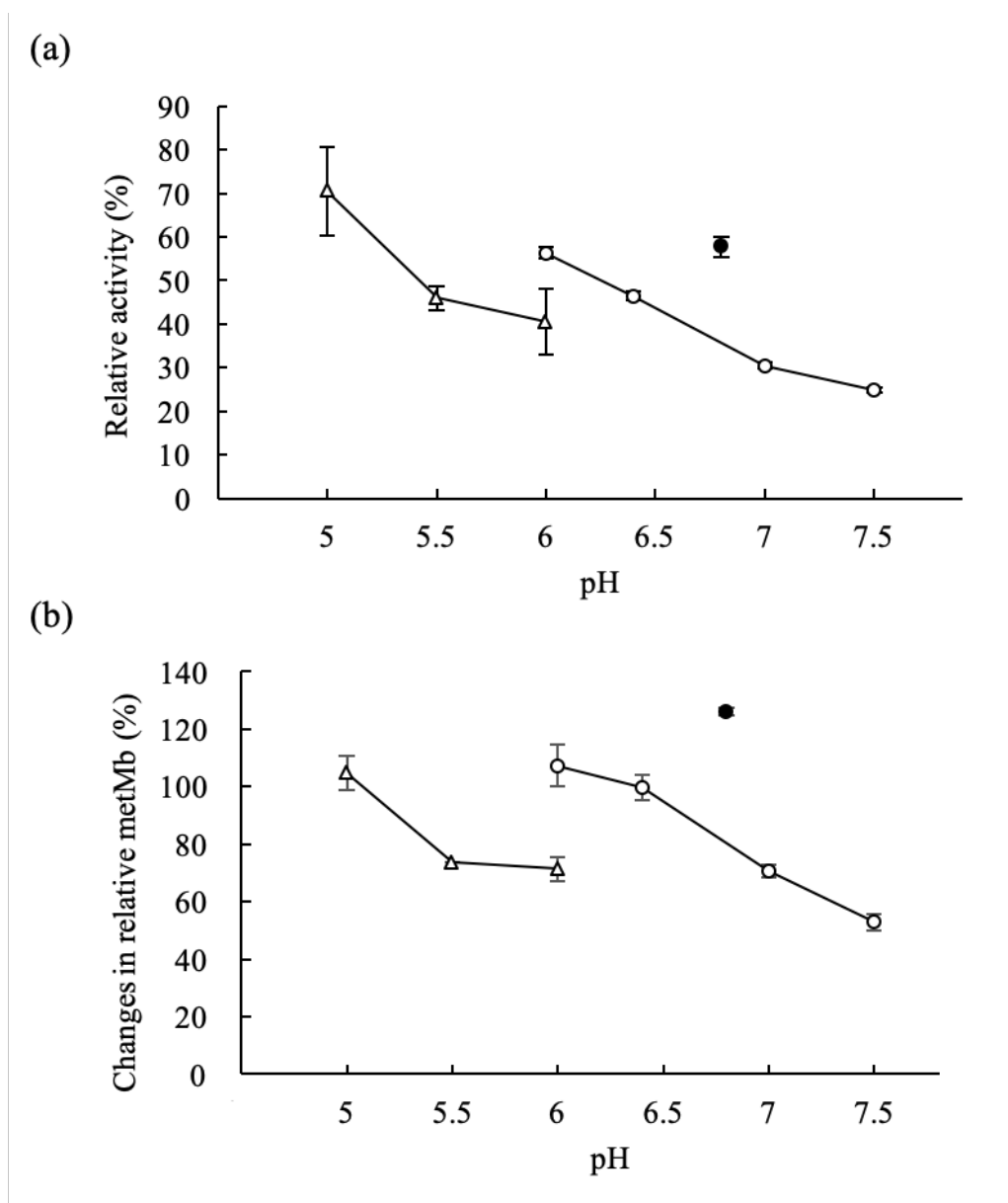
Fig. 5. Effect of pH on metMb reduction. (a) relative activity (%): metMb reductase activity relative to the control; metMb (μmol) was reduced by 1 g of mitochondrial protein per min. (b) changes in relative metMb (%): changes in metMb ratio against total Mb after 10 min reduction relative to the control. All data are shown as averages with S.D. (n=3). Open triangles: citrate buffer; open circles: phosphate buffer; closed circles: Tris-HCl buffer.
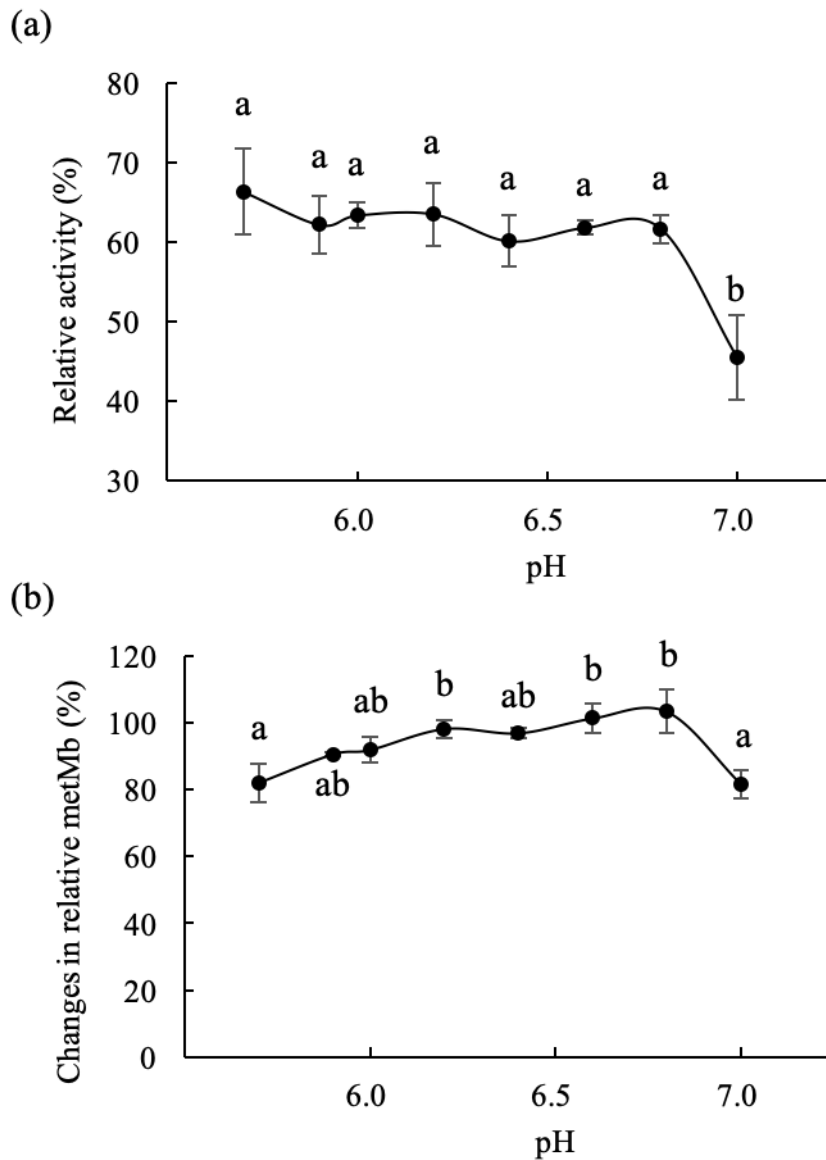
Fig. 6. Effect of pH on metMb reduction. (a) relative activity (%): metMb reductase activity relative to the control; metMb (μmol) was reduced by 1 g of mitochondrial protein per min. (b) changes in relative metMb (%): changes in metMb ratio against total Mb after 10 min reduction relative to the control. The control was measured using citrate buffer (pH 4.9). All data are shown as averages with S.D. (n=3). Different superscripts in the same graph indicate significant differences ($p < 0.05$, Tukey-Kramer).

Effect of NADH concentration

When metMb reduction under different NADH concentrations was measured, metMb % decreased rapidly after NADH addition (Fig. 7), and during the first minute of incubation, similar patterns in the changes of metMb ratio were observed in the range of 0.05-5 mM NADH, except at 0.01 mM NADH, suggesting very low metMb reductase activity at the low NADH concentrations. However, the relative metMb changes (%) maintained the level after 2 min, probably due to the exhaustion of NADH in the reduction system. Furthermore, the relative metMb changes (%) was recovered after approximately 2 min and 5 min of incubation in the presence of 0.05 mM and 0.1 mM NADH, respectively.

When NADH was supplemented into the assay mixture after 10 min incubation (Fig. 8), metMb % showed the similar pattern to that at the beginning of metMb reduction indicating that NADH depletion resulted in increase in metMb reduction.

NADH, as a coenzyme and an electron carrier, is essential for the conversion of ferric Mb to its ferrous form (Bekhit and Faustman 2005). The initial NADH in bluefin tuna muscle was around 130 nmol/g (Pong et al. 2000a), which was higher than that found in beef *longissimus* steaks aged for 3 days (29 nmol/g tissue, Mitacek et al. 2019). Bekhit et al. (2001) reported that the highest metMb reduction was observed at 0.05 mM NADH in the presence of lamb muscle extract. The apparent K_m of metMb reductase for NADH was 2.5×10^{-6} M for the enzyme from the dark muscle of yellowfin tuna (Levy et al. 1985). However, the higher value (24.4×10^{-5} M) was estimated for bluefin tuna reductase (Pong et al. 2000b) as this reductase was probably isolated from

the ordinary muscle. In our study, there was lower reducing activity in the presence of 0.01 mM NADH, whereas no difference in the activity was found in the NADH concentration range of 0.05-1 mM. Hence, 0.05 mM NADH can be considered as a saturation level in the metMb reduction assay. Along with the incease in NADH concentration, the reduction activity of porcine metMb reductase increased to a saturation level at 0.1 mM NADH (Mikkelsen et al. 1999).

The supplemention of NADH during the assay resulted in the resumption of metMb reduction (Fig. 8). It could be hypothesized that regeneration of NADH in the dark muscle during the storage might retain the metMb reduction system. Upon slaughter, most of the glycogen is to be anaerobically converted to lactate which is accumulated in the muscle. Watts et al. (1966) hypothesized that in live muscles, lactate may be oxidized to pyruvate by the lactate dehydrogenase (LDH) and subsequently hydrogen from lactate would be used for NADH production. Consequently, Kim et al. (2006) tested this hypothesis and concluded that non-enzymatic reduction of metMb occurred in an equine lactate-LDH-NAD system, but was inhibited by the exclusion of NAD^+, L-lactic acid or LDH.

In summary, the presence of a metMb reductase system was demonstrated in the mitochondrial fraction from the dark muscle of bigeye tuna, and NADH played a pivotal role in initiation of metMb reduction. This metMb reductase showed higher stability against temperature and pH variations owing to its localization in the mitochondria membrane. Future research should focus on revealing the effect of NADH on the maintenance of metMb reductase activity in postmortem muscle and

determining the importance of the lactate–LDH-NAD system in the enzymatic metMb
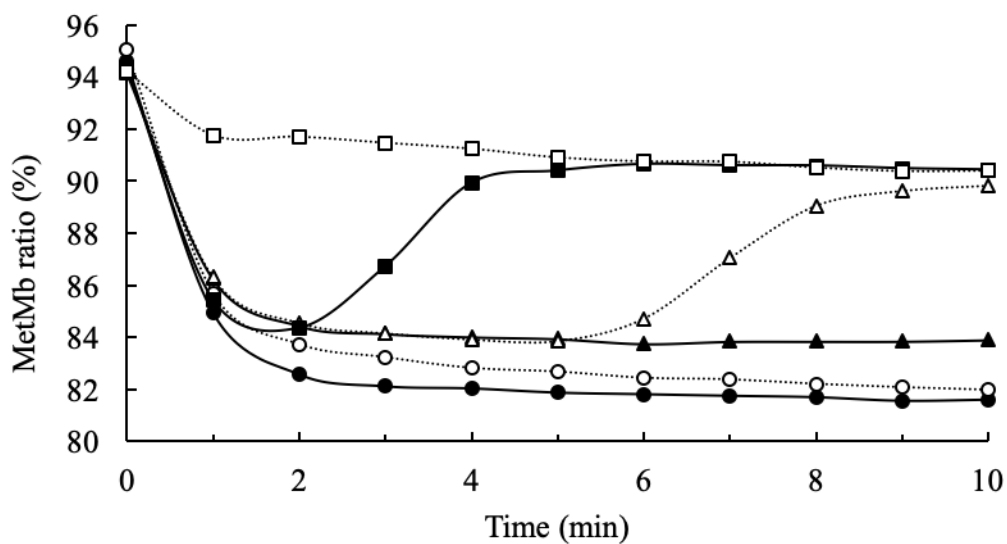
reduction.

Fig. 7. Changes in the metMb ratio under different NADH concentrations. The assay

was carried out in the presence of 5 mM (closed circles), 1 mM (open circles), 0.5 mM

(closed triangles), 0.1 mM (open triangles), 0.05 mM (closed squares), 0.01 mM (open

squares) NADH at 25℃ for 10 min.

31
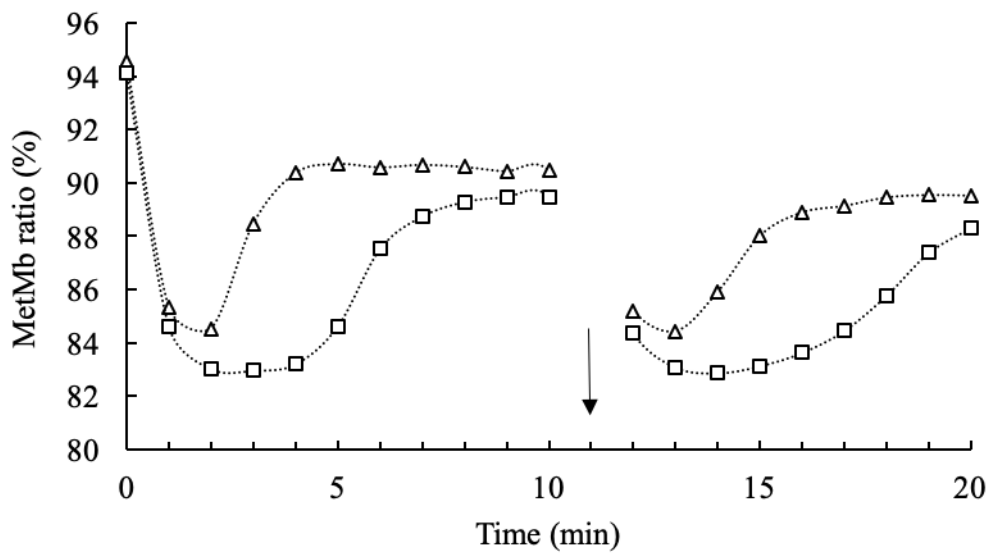
Fig. 8. Changes in the metMb ratio under different NADH concentrations. 0.05 mM (open triangles): metMb reduction under 0.05 mM NADH at 25 ℃ for 20 min but supplemented with the same concentration of NADH as the beginning of reduction at 11 min of incubation; 0.01 mM (open squares): metMb reduction under 0.1 mM NADH at 25 ℃ for 20 min but supplemented with the same concentration of NADH as the beginning of reduction at 11 min of incubation. The arrow indicates when NADH was supplemented.

Chapter 3: Effect of ice storage, freezing and thawing on metMb reducing activity of the mitochondrial fraction

1. Background

In the previous chapter, the presence of a metMb reducing system was demonstrated in the mitochondrial fraction from the dark muscle of bigeye tuna, and NADH played a pivotal role in metMb reduction *in vitro* (Xu et al., 2021). Although previous studies assessed the effect of storage on meat discoloration (Pong et al., 2000a; Hansen et al., 2004; Mancini and Ramanathan, 2014), it seems that evaluation of the metMb reducing activity of tuna muscle has not fully been investigated.

A large amount of fresh meat is stored frozen for long periods and then thawed before being sold as chilled products. Frozen storage can prevent the discoloration of tuna meat effectively, especially at extremely low temperature such as -40 ℃ or lower. In the bluefin tuna meat frozen stored at -20 ℃ to -80 ℃ for one month, followed by iced storage, the lower the freezing temperature, the slower the rate of discoloration (Chow et al., 1988). Similarly, Carballo et al. (2000) revealed that freeze-thaw treatments of raw meat had no effect on lightness, redness, or yellowness of pork batters. However, it is not clear whether metMb reducing activity changes after freezing and thawing.

Therefore, the objective of this chapter was to investigate the metMb reducing activity in the mitochondrial fractions during the ice storage of tuna muscles (dark and ordinary muscles) and to evaluate the metMb reducing activity during the 4 ℃ storages

of the mitochondrial fractions. Furthermore, the effects of freezing and thawing on metMb reducing activity of the mitochondrial fractions were examined in the dark and ordinary muscles of bluefin tuna.

2. Materials and methods

2.1. Materials

Fresh specimens of bluefin tuna (*T. thynnus*) farmed in Kochi Prefecture was purchased at Tsukiji Seafood Market, Tokyo, Japan, and stored on ice for two weeks. Mitochondrial fraction preparation from the dark and ordinary muscles was carried out at 1, 3, 5, 9 and 14 days, respectively. Especially, Day 1 mitochondrial fractions of these two tuna muscles were stored in the refrigerator (4 ℃) for two weeks. MetMb reducing activity was recorded during ice storage of tuna meat and low temperature storage of Day 1 mitochondrial fractions. A part of the muscles was excised and frozen stored at -80 ℃ to clarify the effect of freezing and thawing processes on the metMb reducing activity.

2.2. Chemicals

A 0.45 μm syringe filter was from Minisart NY25X plus. Pierce BCA protein assay kit was from Thermo Fisher Scientific (Fair Lawn, New Jersey, USA). Other chemicals were the same as in Chapter 1.

2.3. Preparation of mitochondrial fraction – Filtration centrifugation method

Mitochondrial fractions were isolated from two tuna muscles according to Lanari and Cassens (1991) and Mancini et al. (2018). In brief, the muscles were homogenized finely with 5 volumes of ice-cold mitochondrial isolation buffer (125 mM sucrose, 125 mM Tris-HCl (pH 7.5), 10 mM KCl, 25 mM EDTA). The suspensions were centrifuged at 1000×g for 20 min. The resulting supernatants were passed through a 0.45 μm syringe filter and centrifuged at 15,000 rpm for 20 min. The pellets were washed twice with mitochondrial suspension buffer (125 mM sucrose, 5 mM Tris-HCl (pH 7.5)) and suspended in the same buffer. All steps were performed at 0 to 4 °C.

2.4. Preparation of mitochondrial fraction – Density gradient ultra-centrifugation method

Preparation of mitochondrial fraction was the same as in Chapter 1.

2.5. Protein concentration determination

Protein concentration determination of the mitochondrial fraction was the same as in Chapter 2.

2.6. MetMb reduction

The reduction activity determination of metMb was similar to that in Chapter 1 according to Halger et al. (1979).

2.7. Statistical analysis

Statistical analysis was performed using Microsoft Excel 2010 (Statcel 4). The significant differences ($p < 0.05$) were determined by using ANOVA.

3. Results and discussion

In this chapter, the filtration centrifugation method (Lanari and Cassens 1991; Mancini et al. 2018) was compared with the density gradient ultra-centrifugation method used in chapter 1. By measuring metMb reducing activity, the mitochondrial fraction yield of the filtration centrifugation method was higher than that of the density gradient ultra-centrifugation method (Table 2). Therefore, in this chapter, the mitochondrial fraction was prepared by the filtration centrifugation method.

Table 2. Effect of extraction method on the yield of the mitochondrial fraction and metMb reducing activity.

Extraction method	Yield (mg/g muscle)	MetMb reductase activity (μmol/min/g protein)
Filtration centrifugation	0.416	74
Density gradient ultra-centrifugation	0.178	71

The changes in the color of the tuna meat during ice storage are shown in Fig. 9. As a result, the browning of tuna meat progressed during the two week storage. Especially from the ninth day, the browning could be confirmed with the naked eyes.
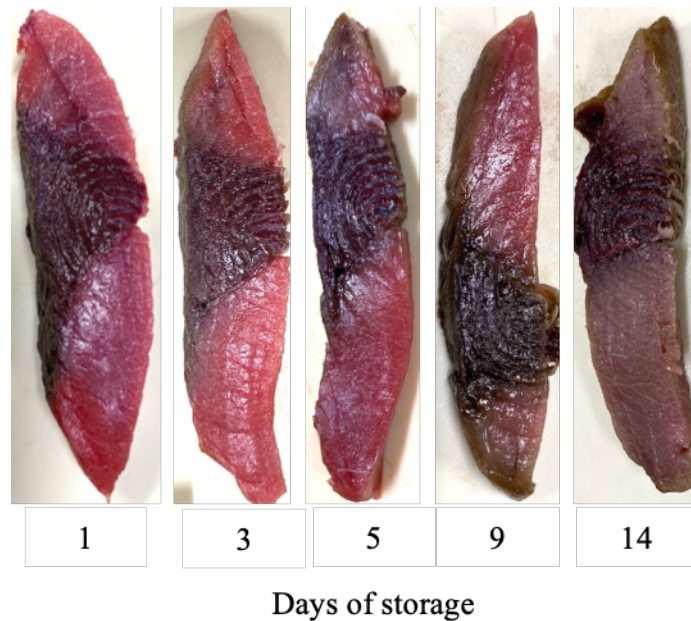

Fig. 9. Changes in the color of bluefin tuna muscle during ice storage.

The effect of ice storage on the yields of mitochondrial fractions from muscles

In the dark and ordinary muscles of bluefin tuna, the effect of ice storage on the yield of mitochondrial fractions was investigated and shown in Fig. 10. Compared to the ordinary muscle, the dark muscle showed twice the yield of mitochondrial fraction. This suggests that there are inherent differences in mitochondrial contents between muscle types, and it was considered that mitochondrial content of the dark muscle was higher than that of the ordinary muscle. During the two weeks of ice storage, the yields of both mitochondrial fractions decreased.

Mitochondria are not distributed in muscle fibers equally. Slow-contracting muscle such as the dark muscle fibers possess a greater mitochondrial content than do fast-contracting fibers (McCormick, 1994). The mitochondrial concentration is higher in type I (predominant red fibers) muscles than in type II (white fibers) (Ke et al., 2017). Consequently, this was in good agreement with higher mitochondrial yield of dark muscle compared with the ordinary muscle of tuna.

Tang et al. (2005a) assessed mitochondrial morphology from postmortem bovine cardiac muscle by electron microscopy at 2 h, 6 h and 60 days postmortem, revealing that mitochondria maintained their structural integrity at 2 h postmortem and prolonged storage caused mitochondrial swelling and breakage. Therefore, decreased mitochondrial yields may have resulted from increased fragility and susceptibility of mitochondria during the postmortem. This was in good agreement with the finding that mitochondrial protein yield decreased during 7 days in beef *longissimus* and *psoas* muscles (Ke et al., 2017). Furthermore, the dark muscle showed greater mitochondrial degradation than the ordinary muscle with increased storage time.
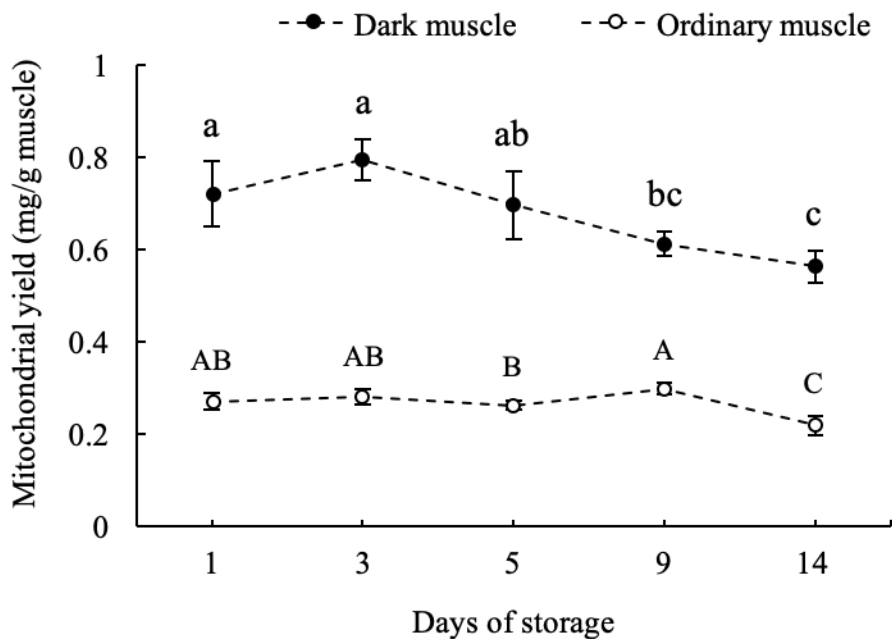
Fig. 10. Changes in the yields of mitochondrial fractions during the ice storage of tuna dark and ordinary muscles. All data are shown as averages with S.D. (n=3). Different superscripts in the same graph indicate significant differences (p <0.05, Tukey-Kramer).

The effect of ice storage on the metMb reducing activity in vitro

The mitochondrial fractions from the dark and ordinary muscles were prepared during the two weeks of ice storage, and the metMb reducing activity was measured in each fraction. The metMb reducing activity of mitochondrial fraction isolated from tuna dark muscle showed no significant difference after two weeks postmortem (Fig. 11(a)). On the contrary, the metMb reducing activity decreased significantly (p <0.05) in the mitochondrial fraction of tuna ordinary muscle (Fig. 11(b)).

Mancini and Ramanathan (2014) reported that decreased color stability in beef

longissimus and cardiac muscles was resulted from the negative effects of storage time on mitochondria-mediated metMb reduction. Similarly, Ke et al. (2017) determined surface metMb reducing activity of beef *longissimus lumborum* and *psoas major* on 0, 3, and 7 days of the display time. Seven days display time made metMb reducing activity of both beef muscles decrease, but the negative effect on metMb reducing activity of *l. lumborum* was greater than that of *psoas major*.

Therefore, there is also general agreement that metMb reducing activity decreases postmortem. Likewise, metMb reducing activity of tuna ordinary muscle was weakened after two weeks postmortem. However, metMb reducing activity of tuna dark muscle was maintained during two weeks postmortem. This difference could be attributed to the differences in species and muscle types.
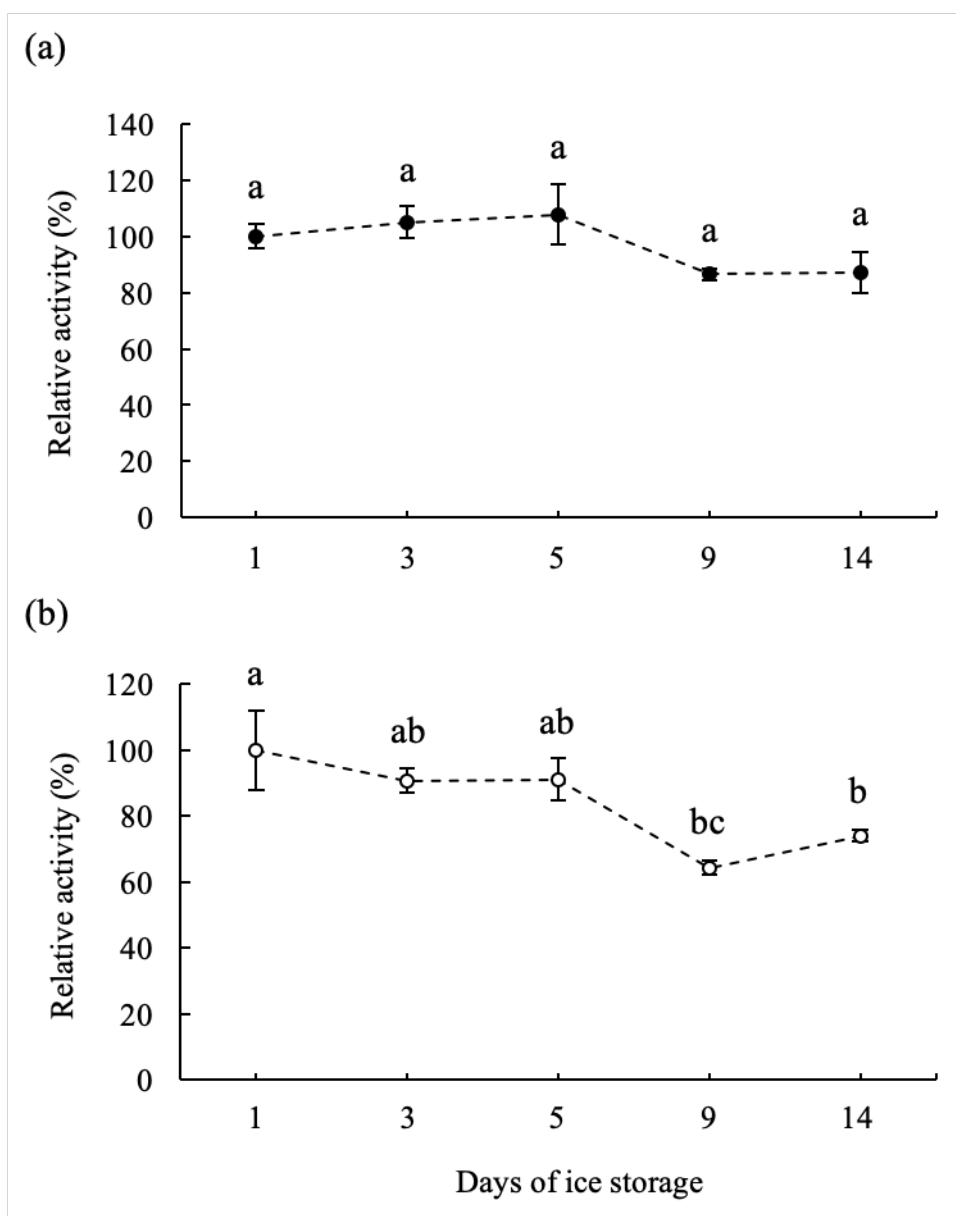
Fig. 11. Changes in the metMb reducing activity of mitochondrial fraction during the ice storage of tuna dark and ordinary muscles. (a) dark muscle; (b) ordinary muscle; relative activity (%): metMb reducing activity relative to the control (Day 1); metMb (μmol) was reduced by 1 g of mitochondrial protein per min. All data are shown as averages with S.D. (n=3). Different superscripts in the same graph indicate significant differences ($p < 0.05$, Tukey-Kramer).

The effect of ice storage of on the metMb reducing activity

Day 1 mitochondrial fractions from tuna dark and ordinary muscles were also stored at 4 ℃ for two weeks, and the metMb reducing activity was measured to evaluate the effect of storage on prepared mitochondrial fractions. Different from the results of muscle storage, the metMb reducing activity of mitochondrial fractions showed no significant change during the ice storage (Fig. 12(a)(b)).

The effect of storage on metMb reducing activity of prepared mitochondrial fractions has been determined for the first time. Stable metMb reducing activity of mitochondrial fractions suggested that storage in sucrose solution well maintains the metMb reducing activity at least for two weeks.
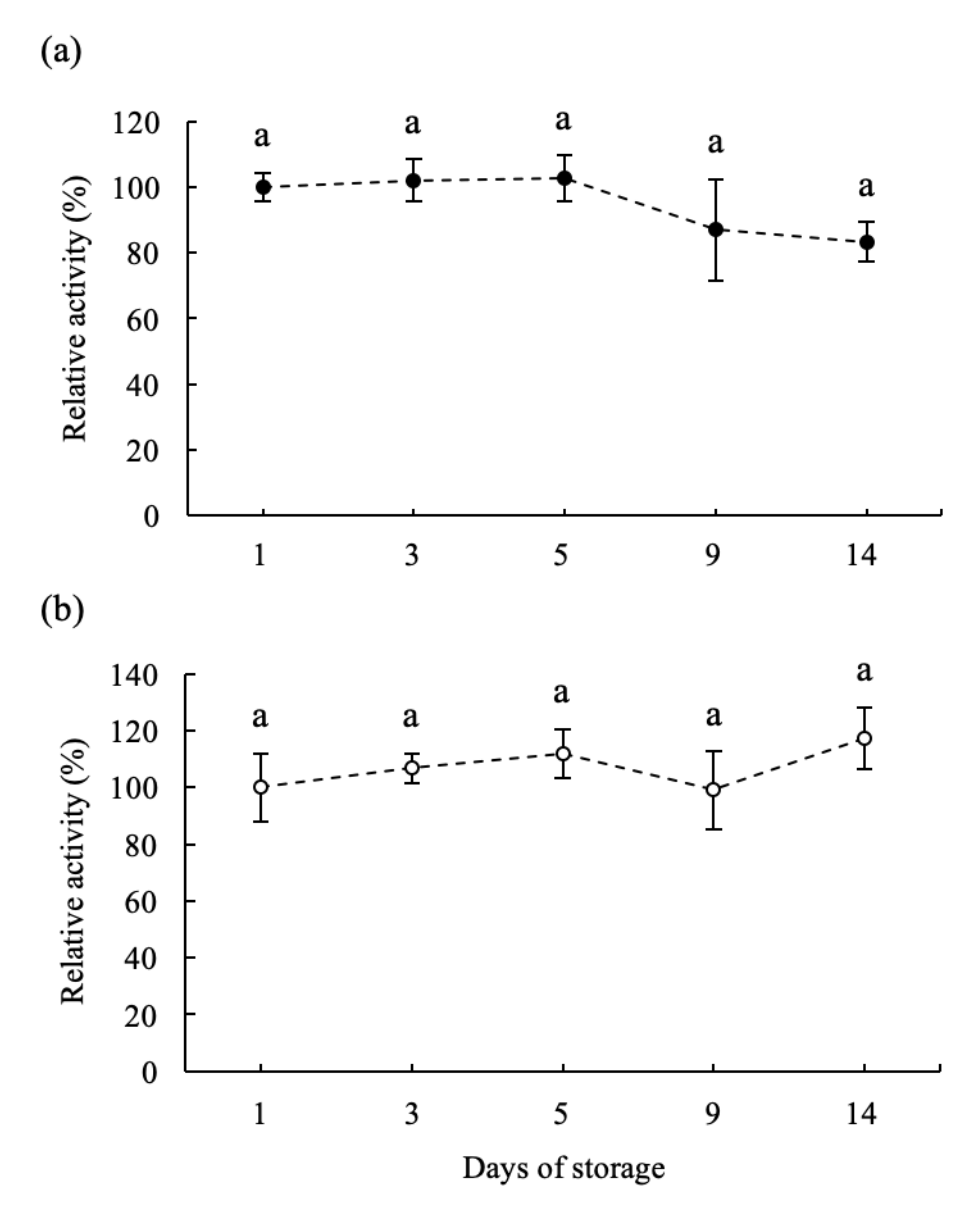
Fig. 12. Changes in the metMb reducing activity of Day 1 mitochondrial fraction from tuna dark and ordinary muscles during the 4 °C storage. (a) dark muscle; (b) ordinary muscle; relative activity (%): metMb reducing activity relative to the control (Day 1); metMb (μmol) was reduced by 1 g of mitochondrial protein per min. All data are shown as averages with S.D. (n=3). Different superscripts in the same graph indicate significant differences ($p < 0.05$, Bonferroni-Dunn).

The effect of freezing and thawing

To investigate the effect of freezing and thawing on metMb reducing activity of the dark and ordinary muscles of tuna, the yields of mitochondrial fractions and metMb reducing activity were measured for frozen and fresh tuna muscles. Compared to the fresh tuna muscles, the frozen dark and ordinary muscles showed significantly ($p < 0.05$) higher yields of mitochondrial fractions (Fig. 13(a)). For metMb reducing activity, the frozen dark muscle revealed higher activity than the fresh dark muscle. Conversely, there was no significant difference between frozen and fresh ordinary muscles metMb reducing activity (Fig. 13(b)).

Similarly, Tang et al. (2006) demonstrated freeze-thaw treatment of mitochondrial preparations had no effect on state IV oxygen consumption rate (OCR), lipid oxidation, electron transport chain (ETC)-dependent metMb reducing activity, or Mb redox stability. In the present study, there was no negative effect of freezing and thawing on the yields of mitochondrial fractions and metMb reducing activity in the dark and ordinary muscles of bluefin tuna. Nevertheless, positive effects were observed in the yields of mitochondrial fractions and metMb reducing activity. It has been reported that freeze-thaw treatment of isolated rat liver mitochondria disrupted mitochondria and increased state IV OCR (Tsvetkov et al., 1985; Tsverkov et al., 1986). Tsvetkov et al. (1985) demonstrated that nonspecific destruction of the inner mitochondrial membrane by freeze-thaw treatment enhanced ATPase activity, thereby increasing state IV OCR. Therefore, mitochondrial structural alterations caused by freezing and thawing process led to increased enzyme release from mitochondria and increased enzymatic activity.
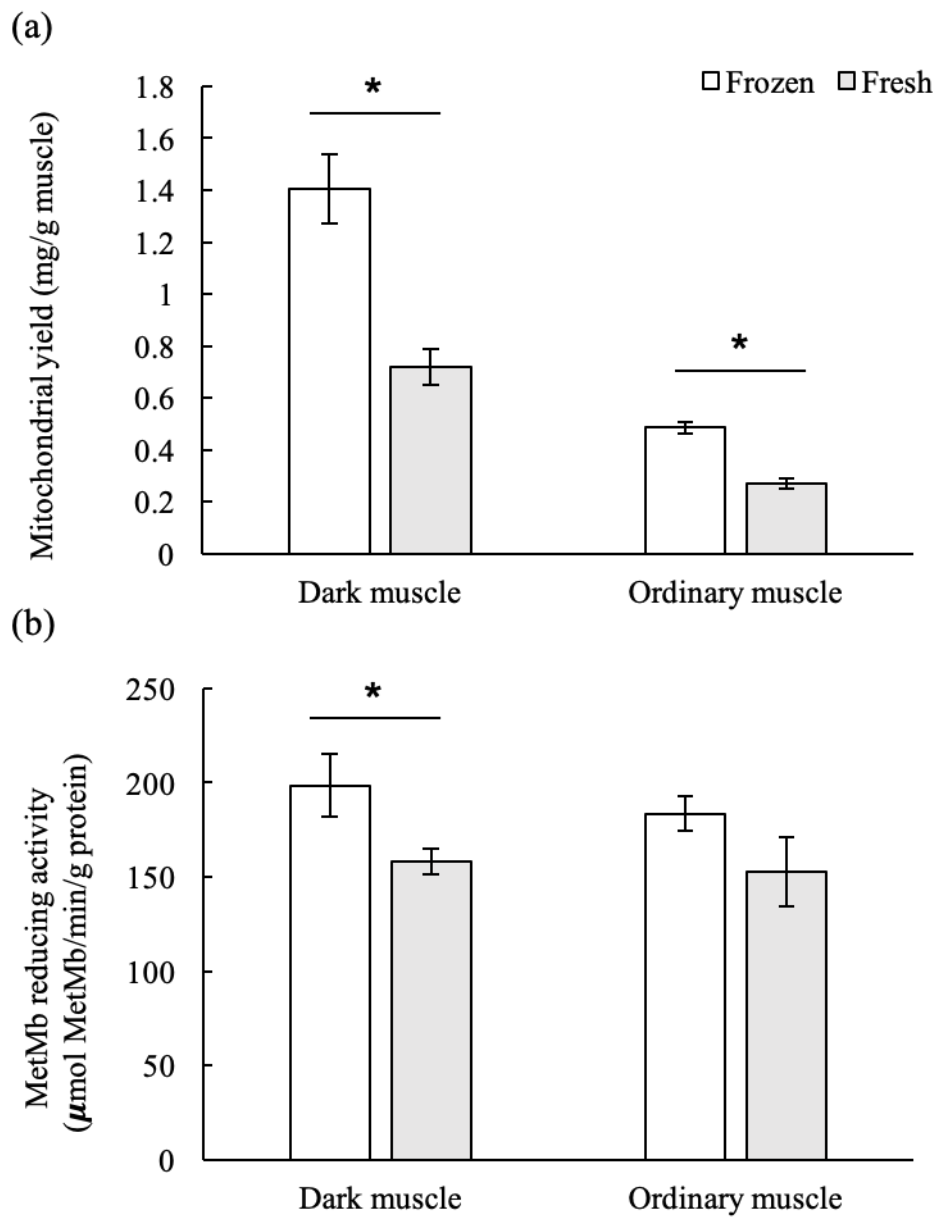
Fig. 13. Effects of freezing and thawing process on the dark and ordinary muscles of tuna. (a) the yields of mitochondrial fractions; (b) metMb reducing activity; metMb (μmol) was reduced by 1 g of mitochondrial protein per min. All data are shown as averages with S.D. (n=3). Asterisks in the same graph indicate significant differences ($p <0.05$, t-test).

To summarize, the *in vitro* metMb reducing activity of the mitochondrial fractions prepared from the dark and ordinary muscles of bluefin tuna was well maintained during the ice storage. In particular, 4 $^{\circ}$C storage in the mitochondrial fractions had no negative effect on the metMb reducing activity. Similarly, the freezing and thawing of tuna dark and ordinary muscles did not show any negative effects on metMb reducing activity. Therefore, it was speculated that low color stability of tuna meat during chilled storage may not be due to the decrease in metMb reducing enzyme activity, but more likely due to the consumption of the metMb reduction substrates.

Conclusion

It was the first time to confirm the presence of NADH-dependent metMb reductase in the mitochondrial fractions of bigeye and bluefin tuna muscles. Although the presence of metMb reducing activity has been demonstrated in various tissues including post-mortem muscle, the extent to which this contributes to maintenance of fresh meat color stability during storage remained unknown. Since metMb reductase is localized in mitochondria, it is highly stable against changes in temperature and pH, and NADH played an important role in metMb reducing activity. However, the main challenge for any role of metMb reducing activity is the expected rapid post-mortem oxidation of its essential cofactor NADH, especially given the normal pH of meat (live muscle: 6.8-7.2, post-mortem muscle: around pH 5.5 due to the accumulation of lactic acid). With sufficient NADH, the activity of metMb reductase was maintained during refrigeration and freezing/thawing. It was shown that the utilization of this enzyme may retard the deterioration of quality associated with browning of meat.

Furthermore, the conflicting conclusions from different researchers arose from examining different reducing systems under a wide range of assay conditions. An accurate standardized method for measuring the reducing activity of metMb needs to be developed to ensure a greater understanding of its relevance to fresh meat.

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