A streamlined isolation method and the autoxidation profiles of tuna myoglobin

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Abstract Determination of the redox state of myoglobin (Mb) gives useful information for evaluating the quality of tuna meat. To attain this purpose, a fast streamlined method has been established basically based on preparative native gel electrophoresis to isolate Mb from the dark muscle of Pacific bluefin tuna. Crude Mb fraction was prepared from dark muscle by ammonium sulfate saturation fractionation and subsequently Mb was purified by preparative native gel electrophoresis under the isoelectric pH of the Mb, resulting in absorption (or trapping) of all the contaminating proteins in the gel. Purified Mb was converted to oxy form with a trace amount of sodium hydrosulfite, and subsequently dialyzed against 50 mM sodium citrate (pH

5.6) or 50 mM sodium phosphate (pH 6.5). The purified tuna Mb was examined for the temperature and pH dependencies of autoxidation using horse Mb as a reference. Tuna Mb was oxidized 2.5–3 times faster than horse Mb irrespective of the pH conditions examined. The highest autoxidation rates both at 0 and 37 °C were observed at pH 5.6. These data were comparable to those obtained for Mbs isolated by conventional chromatographic methods.

Keywords Myoglobin · Streamlined isolation · Preparative electrophoresis · Autoxidation · Tuna

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Introduction

The freshness of tuna meat is essential, especially for sashimi or raw meat products. Generally, determination of tuna meat quality by professional appraisers is based on appearance, odor, and color. It has been known that the meat color of tuna is closely related to the redox state of myoglobin (Mb). Mb is a heme protein found in the striated muscles of vertebrates, especially in slow skeletal muscle (or dark muscle in fish) and heart muscle. Mb functions as a reservoir for temporal oxygen storage in muscles and as a scavenger of nitrogen oxide (Flögel et al. 2010). In muscle foods, Mb greatly contributes to their red coloration. Mb profiles thus can be applied to assess the reliability of tuna meat grading (Nurilmala et al. 2013).

There are three types of Mb derivatives dictated by the redox state of the heme iron. Namely, deoxyMb (the heme iron state being Fe²⁺) is a native purplish-red state without bound oxygen, found in fresh anaerobic muscles. It is changed to bright cherry red oxyMb (the heme iron state being Fe²⁺) when bound to oxygen, and further into

metMb (the heme iron state being Fe^{3+}) of brownish color. Accumulation of metMb results in discoloration (browning) of red meat, thus affecting the commercial values of muscle foods and discouraging consumers' preference (Joseph et al. 2010). For the quality control of muscle food, characterization of purified Mb is essential, especially for red meat including tuna meat, which is susceptible to discoloration because of the higher oxidation rate of Mb compared with the mammalian counterparts.

Teleost fish Mbs consist of 146–147 amino acids, while mammalian ones consist of 153 amino acid residues. It has been known that the tertiary structure of Mb features eight α -helical segments designated A through H from the N terminus. However, the D-helix (the fourth helix from the N terminus) is absent in fish Mbs (Birnbaum et al. 1994). The primary structures of Mbs so far have provided useful information on the relationship between the stability and autoxidation rate (Ueki et al. 2005).

Tuna belong to a group of scombridae fish, having high metabolic rates even at slow swimming speed. Mb is abundant both in the dark and fast skeletal (red) muscles. Although the sequence identities of amino acids of fish Mbs are relatively high among them (> 90%), the stability of Mbs is dependent on species. Tuna Mbs clearly differ in thermostability irrespective of slight amino acid modifications between closely related species and are thus an excellent model protein for investigating the relationship between structure and stability (Ochiai et al. 2009). The temperature-dependency of α -helical content as measured by circular dichroism (CD) spectrometry revealed that the thermal denaturation of tuna Mbs consisted of three stages (at around 20, 46 and 72 °C) suggesting that thermal denaturation of tuna Mb was initiated at quite a low temperature (Ochiai et al. 2010).

The autoxidation of tuna Mb proceeded even during quick freezing and thawing (Chow et al. 1985). Such a behavior of Mb might be supported by the fact that the non-helical region of tuna Mb is susceptible to conformational changes during freezing and thawing (Chow et al. 1989). This could be the reason why fish Mbs are oxidized or discolored more quickly compared with mammalian Mbs. Autoxidation of Mb is also known to be dependent on pH and temperature (Kitahara et al. 1990; Sen et al. 2014). In addition, metMb formation and lipid oxidation are the concomitant processes promoting each other, and thus synergistically deteriorate the quality of meat (Chaijan 2008). Fish, especially tuna, have a large amount of unsaturated lipids, and the oxidized lipids and byproducts accelerate the oxidation of Mb (Alderton et al. 2003; Faustman et al. 2010).

Our previous research has featured the relationship between the stability of tuna Mb and the thermal denaturation pattern (Ueki and Ochiai 2004; Ueki et al. 2005;

Ueki and Ochiai 2006; Ochiai et al. 2010). In addition, acid and alkaline pretreatment accelerated the oxidation rate of tilapia *Oreochromis* spp. Mb (Chow et al. 2009). Concerning the postmortem color stability of tuna meat, pH dependency of the autoxidation rate can be another important factor affecting the rate of discoloration and should be addressed in detail, because muscle pH can reach as low as 5.5 after the death of fish, especially in tuna.

To understand the behavior of Mb during chilled or frozen storage, preparation of Mb as fresh as possible is essential. Many attempts have been made so far to purify Mb using chromatographic methods (Joseph et al. 2010; Thiansilakul et al. 2011; Ueki and Ochiai 2004; Yin et al. 2011; Kobayashi et al. 2014). However, chromatographic techniques are laborious and time consuming to be used routinely, and thus a simple and streamlined method is preferable. By the applying electrophoretic techniques, even fragile proteins such as those participating in photosynthesis, ATP-dependent enzyme or active respiratory supercomplexes could be separated (Seelert and Krause 2008). Isolation of proteins by preparative gel electrophoresis depends on their differential migration rates in the gel column (Margolis et al. 1995). By using the matrices such as agarose or polyacrylamide gel, proteins can be separated from each other (Seelert and Krause 2008). Purification of gliadin was found to be successful when applied to 7% polyacrylamide gels at pH 3.1 (Rumbo et al. 1999). Previous research also demonstrated that embryonic myosin heavy chain isoform was isolated by preparative gel electrophoresis for immunochemical and biochemical studies, resulting in sufficient recovery (Sandri et al. 1999). Because fish Mbs are generally unstable, quick isolation is favorable. Native gel polyacrylamide gel electrophoresis (PAGE) is considered beneficial for its quick isolation, because this method requires much less time than column chromatographic separation.

In the present study, preparative native PAGE was applied to fast streamlined purification of Mb from the dark muscle of bluefin tuna. By using tuna Mb purified thusly, the autoxidation profile under several pH and temperature conditions was investigated in comparison with mammalian (horse) Mb.

Materials and methods

The dark muscle was excised from a specimen of Pacific bluefin tuna *Thunnus orientalis* (~ 30 kg) reared in off the coast of Nagasaki Prefecture, western Japan. The fish was fainted by an electric shock and killed instantly by spiking into the brain and bleeding from the artery beneath the pectoral fin. Subsequently the dark muscle was carefully excised and quickly frozen with dry ice. The frozen muscle

contained in a vinyl bag was partially thawed in tap water just before use in the experiment. All the chemicals used in this study were of reagent grade (Wako, Otsu, Japan). Horse heart Mb was purchased from Sigma Aldrich Chemicals (St. Louis, MA, USA) and used as a control.

Isolation of tuna Mb

The isolation method of tuna Mb was newly established in the present study by the combination of ammonium sulfate fractionation and preparative native PAGE. The following procedures were carried out at 0–4 °C unless otherwise stated. The dark muscle (ca. 2 g) was gently homogenized with a pestle in a mortar (15 cm in diameter) on ice with 2 volumes of ice cold water for 10 min and centrifuged at 2,800 g for 15 min. The supernatant (crude extract of Mb) was filtered through a filter paper (No. 5, Advantec, Tokyo, Japan) followed by filtration through a 0.20- μ m nitrocellulose filter membrane (RC15, Sartorius, Goettingen, Germany). The filtrate was subjected to 40–80% ammonium sulfate saturation and centrifuged at 1100 g for 10 min. The resulting supernatant was brought to 90% ammonium sulfate saturation and centrifuged under the same conditions. The precipitate was dissolved in a small amount of ice-cold water, and dialyzed against electrophoresis buffer (25 mM Tris, 192 mM glycine, pH 8.3) to remove excess ammonium sulfate overnight.

Preparative native PAGE was performed by using a Nativen preparative electrophoresis system (AE-6760L, Atto Co., Tokyo, Japan) equipped with a funnel type disc gel apparatus (37 and 16 mm in diameter at the top and bottom, respectively, and 30 mm in height) (Fig. S1). The gel consisted of 15% separating gel overlaid with 4.5% stacking polyacrylamide gel (ca. 5 mm in thickness). The total volume of acrylamide gel (separating gel) was 25 mL. Two milliliters of dialyzed solution containing ~ 2 g of proteins were mixed with the equal volume of native sample buffer containing 62.5 mM Tris–HCl (pH 6.8), 40% glycerol, 0.01% bromophenol blue (Bio-Rad Laboratories, Hercules, CA, USA) and loaded on to the above acrylamide gel. The apparatus was run for 2 h at 4 °C at a constant current of 15 mA for 3 h. The automatic fractionation step was omitted, because Mb stayed at the surface of the stacking gel because of its pI value being identical to that of the electrode buffer, while all the contaminating proteins which were negatively charged under the electrophoretic condition moved toward the anode (the gel bottom) and were thus absorbed (or trapped) in the separating gel. The presence of Mb above the stacking gel was easily recognized by its unique red color.

SDS-PAGE

To check the purity of Mb, SDS–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out throughout the isolation procedure. Each sample (25 μ L) was mixed with an equal volume of 50 mM Tris–HCl (pH 6.8) containing 0.15% ethylenediaminetetraacetic acid (EDTA), 0.01% bromophenol blue, 4% SDS, 12% glycerol and 1% 2-mercaptoethanol, and were incubated at 95 °C for 3 min. The samples were subjected to a mini slab gel (10 $\times$ 10 cm, 1 mm in thickness) consisting of 15% separating gel and 3% stacking gel. The running buffer consisted of 0.3% Tris base, 1.4% glycine, and 0.1% SDS (w/v). The currents for the stacking and separating gels were 10 and 20 mA, respectively. After separation, the gel was stained with Coomassie Brilliant Blue (CBB) R-250. Molecular weight markers were purchased from Bio-Rad Laboratories (Precision Plus Protein Standards, Hercules, CA, USA).

Two dimensional-polyacrylamide gel electrophoresis (2D-PAGE)

Purified Mb was dissolved in a rehydration solution (7 M urea, 2 M thiourea, 3% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 0.2% ampholyte (pH 3–10), 50 mM dithiothreitol) (Bio-Rad). The treated sample was applied to linear immobilized pH gradient gel (7 cm, pH 3–10, Bio-Rad) at 250 V for 15 min, followed by 4000 V for 2 h, and 20,000 Vh for 6 h. SDS-PAGE for the second dimension was carried out using a 12.5% acrylamide gel after reduction and alkylation treatments of proteins. The gel was stained as described above.

Preparation of oxyMb

OxyMb was prepared from purified Mb according to the method of Tang et al. (2004) with slight modifications. Namely, oxyMb was obtained by adding a trace amount of sodium hydrosulfite (a reducing agent) to Mb solutions and subsequently by gently agitating the solution at 4 °C. The Mb solutions were then dialyzed against 50 mM sodium citrate (pH 5.6) or 50 mM sodium phosphate (pH 6.5 and 7.4) for 2 h to remove the residual hydrosulfite. The final concentration of Mb solution was adjusted to 0.3–0.4 mg/mL with the respective buffers.

Measurement of Mb concentration

To 1 mL of the solution was added 0.5 mL of 25 mM potassium phosphate buffer (pH 7.0), and after gentle mixing, 25 μ L of 5% NaNO₃ was added, followed by the addition of 25 μ L of 1% KCN. After incubation, the

mixture was left at room temperature for 1 min, and the absorbance of the mixture was measured at 540 nm. To calculate the concentration of Mb, the molecular extinction coefficient (11,300) and the molecular weight (15,628) were used (Chen and Chow 2001; Chow et al. 2009) except that the molecular weight (15,900) was replaced by 15,628 which was calculated from the deduced amino acid sequence.

pH dependency of autoxidation rate

The oxyMbs thus prepared were incubated at pH 5.6, 6.5, and 7.4 at 0 °C for up to 7 days and at 37 °C for up to 3.5 h. The absorption spectra of Mb derivatives were measured at specific time intervals from 380 to 780 nm using a spectrophotometer (Jasco V-630 Bio, Tokyo, Japan). The measurement was performed in triplicate and the percentage of metMb formed was calculated according to Tang et al. (2004).

The autoxidation rate (k) was calculated based on the equation as reported by Chow et al. (2004):

$$k = [2 - \log(100 - \text{metMb}\%)]/h,$$

where metMb% stands for the ratio of metMb against total Mb in percent.

Results and discussion

Isolation of tuna Mb

Purified Mb is essential for precise and detailed understanding of its characteristics. In the present study, attempts were made for streamlined purification of tuna Mb Fig. 1 in a short time. For the fast purification of tuna Mb, combination of ammonium sulfate fractionation and native PAGE was found to be successful (Fig. 1). The most

Fig. 1 SDS-PAGE patterns of each purification step of bluefin tuna Mb in a 15% gel. M: molecular weight markers, A: water soluble fraction, B: supernatant of 40% ammonium sulfate saturation, C: precipitate of 40–90% ammonium sulfate saturation, D: purified tuna Mb obtained in the present study

striking advantage of the new method was its ability to make the target protein (Mb) stay in the sample buffer and to absorb all the contaminating proteins into the electrophoresis gel, instead of separating the proteins through the gel matrix.

The conditions for ammonium sulfate fractionation were optimized using the stepwise concentrations of ammonium sulfate saturation, namely 40, 50, 60, 70, and 80%. All the resulting supernatants of ammonium sulfate saturation were brought to 90% ammonium sulfate saturation and then centrifuged. The precipitates were dissolved in a small amount of cold water, and dialyzed against the electrophoresis buffer (25 mM Tris, 192 mM glycine, pH 8.3) to remove excess ammonium sulfate. The fractions of 40, 50, and 60% saturation were found to contain a high amount of Mb (data not shown). Therefore, these fractions were subjected to further purification of Mb by preparative native gel electrophoresis. The purification of Mb from the precipitates of 40–90 and 50–90% saturation was found to be optimal, and the highest yield of Mb was about ~ 9 mg from 2 mL of water soluble protein fraction corresponding to approximately 2 g of dark muscle. Therefore, the 40–90% saturation fraction was used in the subsequent experiments. In the present study as well as in most of the studies to characterize Mbs, a yield of around 10 mg of Mb is considered to be sufficient, and thus only one run of the preparative electrophoresis gives more than a sufficient amount of Mb for the subsequent assay. The total time required for isolation of Mb was about 18 h starting from the extraction, but the time for the purification step was greatly shortened compared with the conventional column chromatographic methods which would have required one more day.

Figure 1 shows the SDS-PAGE patterns of the fractions during the isolation procedure of tuna Mb from 40 to 90% saturation fraction. The water-soluble fraction (lane A) contained a 16 kDa protein corresponding to Mb as well as a large amount of proteins with higher molecular weights. After ammonium sulfate fractionation (40–90% saturation), the Mb band became more intense (lane C). The contaminating proteins were then removed by preparative electrophoresis, resulting in a single band (approximately 16 kDa) of Mb (lane D) at the final stage of purification. Therefore, the yield of Mb in the present study was estimated to be about 53%.

2D-PAGE further demonstrated the high purity of Mb (Fig. 2). 2D-PAGE gel gave only one spot, corresponding to Mb, demonstrating the homogeneity of purified Mb. The isoelectric point (pI) of purified Mb estimated based on the position of the spot, was found to be at around 9.0, quite close to the calculated value (8.99) based on its deduced amino acid sequence (DDBJ/EMBL/Gen Bank databases with the accession number of AF291836).

Fig. 2 Two dimensional PAGE pattern of purified bluefin tuna Mb

In this study, the native PAGE was carried out to purify tuna Mb following ammonium sulfate fractionation. The SDS-PAGE pattern for the purification steps without ammonium sulfate fractionation gave an additional protein band of about 29 kDa (data not shown). This protein showed the same isoelectric point as that of Mb in the 2D-PAGE (data not shown), and was thus considered to be a Mb dimer formed by aggregation. Therefore, ammonium sulfate fractionation was essential to prepare Mb of high purity. However, regarding the pH value of the electrode buffer used for the native PAGE, pI of Mb might differ depending on the animal species. Therefore, the pH value should be adjusted exactly to the pI value of the target Mb to obtain higher purity.

Autoxidation rate of Mb at 0 °C

DeoxyMb that was purple-red in color was obtained immediately after addition of sodium hydrosulfite. Dialysis of Mb against 50 mM sodium citrate buffer (pH 5.6) or sodium phosphate buffer (pH 6.5 and 7.4) resulted in partial conversion of a deoxy form to an oxy form and removal of excess sodium hydrosulfite.

OxyMb was changed to a metMb form with a prolonged time of incubation. The changes in the metMb ratio of both tuna and horse Mbs during incubation at 0 °C are presented in Fig. 3. Autoxidation of both Mbs proceeded as a first-order reaction irrespective of pH examined. Compared with horse Mb, autoxidation rates of tuna Mb were found to be 2.5–3 times higher at all pHs examined (Fig. 4a). This result is in good agreement with the previous research showing that tuna Mb is approximately 2.5 times more susceptible to oxidation than mammalian Mbs (Livingston and Brown 1981). The pH conditions affect changes in Mb formation, indicating that pH 6.5 was the optimal for minimizing its autoxidation (Chow et al. 1985). The autoxidation rate was found to be the highest at pH 5.6 for both tuna and horse Mbs. These results show that the

Fig. 3 Changes in the metMb ratio of bluefin tuna and horse Mbs during incubation at 0 °C under different pHs. **a** 50 mM sodium citrate buffer (pH 5.6); **b** 50 mM phosphate buffer (pH 6.5); and **c** 50 mM sodium phosphate buffer (pH 7.4). Filled and open circles represent bluefin tuna and horse Mbs, respectively. The results are provided as the mean values with standard deviations ($n = 3$)

Fig. 4 pH dependency of the metMb formation rate. **a** 0 °C; **b** 37 °C. Filled and open circles represent bluefin tuna and horse Mbs, respectively

stability of both Mbs was the highest at pH 6.5 followed by pH 7.4 and 5.6, respectively.

The results on the pH dependency of Mb autoxidation agreed well with those of previous reports (Chow et al. 1985, 1987). The highest value of free energy for unfolding of tuna Mb was found at around pH 6.5 (Chow et al. 1989), supporting the results of this study. It has been reported that the stability of the heme and its affinity for globin decreases in an acidic environment (Livingston and Brown 1981). Exposure of the heme to the medium seems to promote the formation of metMb even at an incubation temperature as low as 0 °C. In addition, the autoxidation rate of tuna Mb was clearly higher than that of horse Mb under all pH values examined. Under near neutral pH conditions, the structural stability of Mb was considered to be maintained as estimated by the lower autoxidation rate at pH 6.5 and 7.4 for both Mbs.

Autoxidation rate of Mb at 37 °C

The autoxidation profiles of tuna and horse Mbs at 37 °C (close to the body temperatures of both animals) under various pH conditions are shown in Fig. 5. The autoxidation rate of Mb also followed a first-order reaction as previously reported (Kitahara et al. 1990; Chen and Chow 2001). The rate for tuna Mb was slightly higher than that of horse Mb under all pH conditions. The rate for tuna Mb was the highest at pH 5.6, followed by those at pH 6.5 and 7.4. After 3.5 h of incubation, the rates at pH 5.6, 6.5, and 7.4 were found to be 91.9, 72.0, and 43.1%, respectively. The highest rates of both Mbs were obtained at pH 5.6, followed by pH 6.5 and 7.4 (Fig. 5b, c). It was noteworthy that the autoxidation rates of both Mbs showed similar values at pH 7.4. Under a near neutral pH condition, the structural stability of Mb is likely to be maintained as shown by the lower autoxidation rates at pH 6.5 and 7.4 for both Mbs.

Compared with horse Mb, the autoxidation rate of tuna Mb was higher at pH 5.6 and 6.4, but not at pH 7.4 (37 °C). The highest autoxidation rate was obtained at pH 5.6 for both Mbs, followed by pH 6.5 and 7.4. To minimize Mb autoxidation, it is quite reasonable to keep the temperature close to 0 °C and near neutral pH (6.5) throughout the purification. This environment would be very effective in preventing Mb autoxidation, in other words, discoloration or quality deterioration of tuna meat.

In the present study, the autoxidation rate of bluefin tuna Mb was calculated to be 0.071/h at pH 7.4, while Madden et al. (2004) reported that of yellowfin tuna Mb was 0.088/h at pH 7.5. They compared the autoxidation rates of Mbs from four fish species: namely, yellowfin tuna (a homeothermal teleost), zebrafish (a stenothermal tropical teleost), mackerel (a eurythermal teleost), and Antarctic species *Notothenia*

Fig. 5 Changes in the metMb ratio (%) of bluefin tuna and horse Mbs during incubation at 37 °C. **a** 50 mM sodium citrate buffer (pH 5.6); **b** 50 mM phosphate buffer (pH 6.5); **c** 50 mM sodium phosphate buffer (pH 7.4). Filled and open circles represent bluefin tuna and horse Mbs, respectively. The results are provided as the mean values with standard deviations ($n = 3$)

coriiceps (a stenothermal teleost). The autoxidation rate of bluefin tuna obtained in this study (0.071/h) was lower compared with those of zebrafish (0.221/h), mackerel (0.259/h), and *N. coriiceps* (0.441/h).

Conclusion

The isolation method introduced in the present study require short time for the purification and omit laborious procedures. The proposed method is beneficial for improving the quality of prepared Mb, especially for fish Mbs which are known to show lower stability compared with mammalian counterparts. Clear temperature and pH dependencies of Mb autoxidation were observed as has been reported for the other Mbs. The present study showed that isolation of Mb by the methods as described above provided Mb that behaved in the same way as in the previous studies on Mb autoxidation.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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