論　文　の　内　容　の　要　旨

水圏生物科学専攻
平成　22 年度博士課程　入学
氏　　名　　マーラ　ヌリルマーラ
指導教員名　　潮　秀樹

論文題目 Studies on the structural changes of myoglobin in tuna meat discoloration (マグロ筋肉の変色におけるミオグロビンの変性に関する研究)

Myoglobin (Mb) is a water-soluble and oxygen-binding hemoprotein. Mb is found mainly in the slow skeletal and heart muscles of vertebrates. Scombridae fish such as tuna have abundant Mb both in slow and fast skeletal muscles (also referred to as ordinary muscle, light muscle or white muscle). Mb consists of 7-8 α-helical segments, designated A through H from the N terminus. It is well established that the main function of Mb is to store temporarily oxygen in the muscle for facilitation of respiration, though new functions of Mb have also been recently reported such as scavenging activity of nitrogen oxide to respond hypoxic environments in order to maintain cellular homeostasis.

It has been considered that the primary structures of Mbs have correlations with their stability. Previous studies revealed that structural stabilities of scombridae fish Mbs clearly differ among the species, where skipjack tuna *Katsuwonus pelamis* Mb was the most thermostable and bullet tuna *Auxis rochei* Mb showed the lowest stability. Skipjack Mb exceptionally contained 146 amino acid residues, while those of other teleost fish so far reported consist of 147 amino acids. However, it is still unclear which factor(s) determines the stability of Mbs. Thus, determining the primary structure would provide useful information on the stability of Mbs. Tuna Mb can be an excellent model fish in this kind of studies.

Mb gives rise to three types of derivatives depending on the redox state of the iron atom in the heme, namely, deoxyMb, oxyMb, and metMb. Interconversion among Mb derivatives is affected by several factors such as temperature, pH, metMb reducing activity, and lipid oxidation. Meat discoloration is closely related with the proportion of the derivatives. Autooxidation rate of Mb influences discoloration of meat, and also is closely

related with the stability of Mb. Previous studies reported that fish Mbs are less stable compared to mammalian Mbs. However, the molecular mechanism regarding their denaturation and autooxidation still remains unclear. In the present study, attempts have been made to characterize Mbs from the bluefin tuna species and their thermal denaturation profile. Assessment of quality evaluation of tuna meat was also carried out based on the properties of Mb. The thesis consists of the following three chapters.

Chapter 1

The primary structure of southern bluefin tuna *Thunnus maccoyii* Mb has been elucidated by molecular cloning techniques. The cDNA of this tuna encoding Mb contained 776 nucleotides, with an open reading frame of 444 nucleotides encoding 147 amino acids. The 5' and 3' non coding regions were 74 bp and 258 bp, respectively. The primary structure has been submitted to DDBJ/EMBL/GenBank databases with the accession number of AB592346. The amino acid sequences as well as the nucleotide sequences were identical to those of bluefin tuna *T. thynnus* and *T. orientalis* Mbs, suggesting that Mbs from these tuna species share the same structural and functional properties.

A phylogenetic tree was constructed based on the deduced amino acid sequences by neighbor-joining methods. The sequences of sea hare and sperm whale Mbs were taken as outgroups to root the tree. Southern bluefin tuna Mb formed one clade with the other tuna Mbs. In addition, scombridae Mbs formed an independent cluster. Mb from Atlantic blue marlin, a member of scombridae, was found to be the most distant from the other scombridae species Mbs. Moreover, the identity of amino acid sequence was relatively high among scombridae fish (82-100%).

Based on its deduced sequence, the isoelectric point and molecular weight of southern bluefin tuna Mb were calculated to be 8.99 and 15628, respectively. Hydropathy plot revealed that the segment E was less hydrophobic, while the lower hydrophobicity of the segment F could be due to the presence of His89. Homology modeling based on the tertiary structure of blackfin tuna Mb (PDB ID 2NRL) as a template demonstrated that southern bluefin tuna Mb has a very similar structure to those of other fish Mbs.

Chapter 2

In order to understand the stability of tuna Mb, an attempt has been made to elucidate the autooxidation profiles under several pH and temperature conditions. Firstly, a fast stream-lined method was developed basically based on ammonium sulfate fractionation and preparative native gel electrophoresis to purify the Pacific bluefin tuna Mb. Namely, the water soluble fraction of dark muscle was subjected to ammonium sulfate fractionation, and the fraction (40-90% saturation) was subsequently applied to the preparative electrophoresis. Under the electrophoretic condition, because of the identity of the buffer pH and the isolectric point, only Mb stayed on the gel top, whereas the other proteins entered the gel, resulting in the pure fraction of native Mb.

The Mb preparation gave a single band in SDS-PAGE gel. Two-dimensional PAGE gave only one spot corresponding to Mb. The absorbance spectra of Mb derivatives (deoxy, oxy, met forms) were then measured for tuna Mb and horse Mb as a control. The extinction coefficient for the β–maximum (at 541 nm) of tuna oxyMb was higher than that of α-maximum at 577 nm. The maximum absorption of horse Mb shifted to the higher wavelength by 1 nm for all the derivatives, while the α-maximum of oxyMb (581 nm) shifted to the longer wavelength by 4 nm.

To investigate autooxidation profiles, oxyMbs were prepared from purified tuna and horse Mbs which had been dialyzed in advance against 50 mM sodium citrate (pH 5.6) or sodium phosphate buffer (pH 6.5 and

7.4) at 4°C after reduction by addition of sodium hydrosulfite. The final concentration of Mb was adjusted to 0.3-0.4 mg/ml with the respective buffers, and then incubated at 0°C for up to 7 days and at 37°C for up to 3.5 h. As a result, the autooxidation of these Mbs proceeded as a first order reaction irrespective of pH and temperatures examined. The highest autooxidation rate at 0°C was observed at pH 5.6, followed by at pH 7.4 and 6.5 for both tuna and horse Mbs for up to 7 days. Tuna Mb autooxidized 2.5-3 times faster than horse Mb under all the pH conditions examined. However, the highest autooxidation rates of both tuna and horse Mbs at 37°C were obtained at pH 5.6 followed by at pH 6.5 and 7.4, although the autooxidation rate of tuna and horse Mbs were similar at pH 7.4.

The denaturation profiles of tuna Mb was compared to that of horse Mb. Percentage Mb denaturation (PMD) was investigated under combination of pH 5.6, 6.5, 7.4 and temperature 70, 75, 80°C. In addition, to unveil the stability of tuna Mb, PMD was measured at 55, 60, and 65°C at pH 6.5. Under all the pH examined at 75 and 80°C, tuna Mb was almost completely denatured after 10 min of incubation as demonstrated by a high PMD value (>90%). The denaturation rates of tuna Mb were represented by the changes of natural logarithm of residual concentration of Mb, resulting in a biphasic first order reaction. The denaturation proceeded at 55 and 60°C, although PMD value increased very slowly, reaching less than 10%. At 65°C, PMD gradually increased to 32.3% after 10 min. On the other hand, horse Mb was very stable at 6.5 and pH 7.4 at all temperatures examined except at 80°C at pH 6.5. However, PMD of horse Mb was gradually increased at 75 and 80°C only at pH 5.6.

Chapter 3

An attempt was carried out to investigate the relationship among commercial sensory evaluation grades of yellowfin tuna *Thunnus albacores* meat with Mb properties including Mb derivatives ratio, color values, and Mb extractability. The SDS-PAGE pattern of the water soluble fraction was also examined to monitor the deterioration extent of meat quality.

The four quality grades, namely, excellent, good, acceptable, and "not acceptable" as judged by the professional appraiser were compared based on the above parameters. The ratio of Mb derivatives was determined based on the visible absorption spectra of the meat water extract. As a result, the grade of meat was found to be significantly correlated with metMb ratio (%) ($P < 0.01$). MetMb ratio of the "not acceptable" grade meat was significantly higher than the other samples of the higher grade (65 %). In contrast, the highest ratio of the oxyMb was found in the excellent meat, followed by the good, acceptable, and "not acceptable" meats in this order. Thus, both metMb and oxyMb ratios could be good parameters for the quality of tuna meat. As far as the deoxyMb ratio is concerned, no significant correlation with the meat quality was observed.

The extractability of Mb was reduced in the lower grade meat with the significance level of $P < 0.01$. Therefore, it is likely that Mb was partially denatured, resulting in the insolubilization of Mb especially in the meat of the lower grade. Color measurement revealed significant differences in the a^* value between the different grade meat, but essentially no difference in the L^* and b^* values. Both the a^* value and redness index (a^*/b^*) showed high correlation coefficients with metMb ratio.

Since the high correlation of both a^* value and redness index against metMb ratio (%) was found, these parameters can be used as indicators for grading of tuna meat. On the other hand, the meat of different grade showed distinguishable patterns in the SDS-PAGE gel. Especially, the band below Mb was not found in the excellent grade meat, unlike the other grades, suggesting that this band can be used as a parameter of

deterioration. These results demonstrated that the quality ranking of yellowfin tuna meat as determined by sensory tests by the professional appraiser was quite reliable.

As described above, the amino acid sequence of southern bluefin tuna Mb was reported in the present study for the first time. Phylogenetic analysis and homology modeling of the structure revealed the typical profiles of this Mb as scombroid fish Mbs. Then the stability of bluefin tuna Mb was examined under various pH and temperature conditions taking PMD as a parameter. The results obtained showed that the Mb becomes unstable at lower pH and higher temperature, and the stability was much lower than that of horse Mb as a control. Finally, the reliability of commercial evaluation of tuna meat quality was assessed basically based on the properties of Mb. The results showed the quality grading was quite reliable. Through the present assessment, excellent markers for precise grading of tuna meat quality were found.

The present study not only succeeded in obtaining new insights to the properties and stability profiles of tuna Mb, and but also gave useful information for quality control of tuna meat. The study seems to have made a contribution to the science of fish Mbs and effective utilization of tuna. Further studies are required to reveal detailed mechanism of Mb denaturation and establish the conditions to prevent discoloration of tuna meat.