CHAPTER 9

INTERACTION BETWEEN FISH MYOGLOBIN AND MYOFIBRILLAR PROTEINS MEDIATED BY FREEZING PROCESS AND ALDEHYDES

9.1 Abstract

Interaction between fish myoglobin and natural actomyosin (NAM) extracted from fresh and frozen fish was compared. The bound myoglobin content in the mixture of myoglobin-NAM extracted from frozen fish was relatively higher than that extracted from fresh fish (P<0.05). However, no differences in bound myoglobin content in myoglobin-NAM prepared from frozen whole fish and fillet were observed (P>0.05). The formation of metmyoglobin in myoglobin-NAM mixture was generally higher than that observed in the control myoglobin (P <0.05) and the highest metmyoglobin content was found in the mixture of myoglobin-NAM extracted from frozen whole fish (P<0.05). Myoglobin-NAM interactions likely occurred via both disulfide and non-disulfide bonds. The effect of different aldehydes on the interaction between fish myoglobin and NAM was also studied. The binding of NAM to myoglobin was higher in the presence of aldehyde (P<0.05). The degree of binding varied with aldehyde types. The highest bound myoglobin content of NAM-myoglobin mixture was found in the presence of hexenal (P <0.05). The addition of hexenal to myoglobin and NAM-myoglobin mixture resulted in the greater oxymyoglobin oxidation than did hexanal (P<0.05). The whiteness of both washed NAM and washed NAM-myoglobin mixture decreased with the addition of aldehydes, especially hexenal (P<0.05). Without myoglobin, Ca^{2+}-ATPase activity of hexenal added NAM was lower than that of that added with hexanal (P<0.05). However, no differences in Ca^{2+}-ATPase activity between hexanal and hexenal treated sample when myoglobin was combined with NAM (P> 0.05). SDS-PAGE revealed that both disulfide and non-disulfide covalent linkage contributed to the cross-linking between myoglobin and myofibrillar proteins induced by aldehydes. Hexenal more likely induced the cross-linking via non-disulfide bonds.

9.2 Introduction 14

Myoglobin and hemoglobin play an essential role in the whiteness, which is one of factors determining the quality of surimi gel (Chen, 2002). In general, hemoglobin is lost rather easily during handling and storage, whereas myoglobin is retained by the intracellular structure (Haard *et al*., 1994). Normally, both heme proteins in fresh fish can be removed during the washing process, leading to increased whiteness of resulting surimi. However, heme proteins become less soluble as the fish undergo deterioration (Chen, 2003). Denaturation of the myoglobin and/or myofibrillar proteins, before or during processing, can also enhance their interaction, thereby causing the discoloration of the surimi (Lanier, 2000). After capture, fish are normally kept in ice or frozen on board prior to unloading (Emilia and Santos-Yap, 1995). During this stage, discoloration of muscle can occur and binding of pigments to muscle also takes place. Fish myofibrillar proteins are highly susceptible to physicochemical and structural modifications caused by freezing and frozen storage, leading to intramolecular conformational rearrangements and intermolecular aggregation (Owusu-Ansah and Hultin, 1986). Benjakul and Bauer (2001) reported that quality deterioration of frozen stored fish was due to the osmotic removal of water, denaturation of protein and mechanical damage.

Apart from the denaturation of protein, lipid oxidation is one of the major problems in stored fish (Benjakul and Bauer, 2001; McDonald and Hultin, 1987). Fatty fish may undergo rapid lipid oxidation during refrigerated storage due to the high content of polyunsaturated fatty acids (Apgar and Hultin, 1982). Chaijan *et al*. (2006) reported that both lipolysis and lipid oxidation occurred progressively in sardine (*Sardinella gibbosa*) during 15 days of iced storage. Lipid oxidation generates a wide range of secondary aldehyde products including n-alkanals, trans-2-alkenals, 4-hydroxy-trans-2-alkenals, and malonaldehyde (Esterbauer *et al*., 1991). Secondary products from lipid oxidation, especially aldehydes, can induce the myofibrillar proteins cross-linking, resulting in the structural and functional changes in these proteins (Toroni *et al*., 2002; Li and King, 1999). Furthermore, aldehyde lipid oxidation products can alter myoglobin stability (Lynch and Faustman, 2000). Covalent modification of

equine, bovine, porcine and tuna myoglobin by 4-hydroxynonenal has been demonstrated (Faustman *et al*., 1999; Phillips *et al*., 2001a, b; Lee *et al*., 2003a, b).

The degradation and/or denaturation of muscle protein during frozen storage and the formation of aldehyde lipid oxidation products during post-mortem handling and storage may negatively affect the color of processed fish muscle, presumably due to interaction of myoglobin with muscle proteins. Therefore, the objectives of this study were to investigate the interaction between yellowfin tuna myoglobin and myofibrillar proteins extracted from fresh and frozen bluefish and to study the effect of aldehyde lipid oxidation products on the interaction between myoglobin and myofibrillar proteins in a model system at refrigerated temperature (4°C).

9.3 Materials and Methods

Chemicals

Sephacryl S-200HR, sodium dodecyl sulfate (SDS), □-mercaptoethanol (□ ME), Triton X-100, Tris(hydroxymethyl)-aminomethane, sodium hydrosulfite, sodium phosphate, potassium chloride and bicinchoninic acid (BCA) protein assay kit were obtained from Sigma (St. Louise, MO, USA). Ammonium sulfate was purchased from Fisher Scientific (Fair Lawn, NJ, USA). All chemicals were of analytical grade.

Fish sample

Bluefish (*Pomatomus saltatrix*) with an average weight of 1.18 kg (n=5) and yellowfin tuna (*Thunnus albacares*) loins (8.4 kg) were obtained from MC Fresh Inc. (Narragansett, RI, USA). The fish, off-loaded approximately 24-36 h after capture, were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Animal Science, University of Connecticut within 1-2 h. Bluefish were divided into two groups, fresh and frozen fish. For the fresh fish, whole bluefish were immediately washed, filleted, skinned and subjected to natural actomyosin (NAM) extraction. For the frozen samples, the whole fish or fillet were stored at -20°C in an air-blast freezer. After one month of storage, ordinary muscles were

collected and used for NAM preparation. The longissimus muscles of yellowfin tuna were cut into 0.2 kg pieces and stored under vacuum packaging at -20°C until required for myoglobin isolation.

Extraction and purification of myoglobin from tuna muscle

Extraction and purification of myoglobin were performed according to the method of Trout and Gutzke (1996) with a slight modification. The yellowfin tuna longissimus muscle (100 g) was minced coarsely and homogenized in a Waring blender with 300 ml of cold extraction buffer (10 mM Tris-HCl, pH 8.0 containing 1 mM EDTA and 25 g/l Triton X-100) for 1 min at high speed. The homogenate was centrifuged at 5,000×*g* for 10 min at 4°C using a RC-5B centrifuge (Sorvall, Norwalk, CT, USA). The supernatant was filtered through two layers of cheesecloth and the pH was adjusted to 8.0 using 0.2 M NaOH. Solid ammonium sulfate was added to the filtrate to 65% saturation and the suspension was stirred for 60 min at 4°C. The suspension was centrifuged at 18,000×*g* for 20 min at 4°C and the pellet was discarded. The resulting supernatant was filtered through two layers of cheesecloth, adjusted to pH 8.0 using 0.2 M NaOH and brought to 100% saturation with solid ammonium sulfate. This suspension was stirred for 60 min at 4°C and then centrifuged at 20,000×*g* for 60 min at 4°C. The resulting soft myoglobin-containing pellet was dissolved in a minimal volume of cold 5 mM Tris-HCl buffer containing 1 mM EDTA, pH 8.0, hereafter referred to as starting buffer. The mixture was then dialyzed against 10 vol starting buffer for 24 h at 4°C. The dialysate was immediately applied to a Sephacryl S-200HR column (2.5 × 100 cm) which was previously equilibrated with starting buffer. The separation was conducted at a flow rate of 60 ml/h and 5-ml fractions were collected.

During purification, the fractions obtained were monitored at 280 nm and 540 nm using a Shimadzu UV-2100 spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD, USA). The fractions with absorbance at 540 nm were pooled and used as "purified tuna myoglobin".

Preparation of oxymyoglobin

Oxymyoglobin was prepared by hydrosulfite-mediated reduction of purified tuna myoglobin (Brown and Mebine, 1969). Residual hydrosulfite was removed by dialysis of the sample against 10 vol of cold 10 mM phosphate buffer, pH 6.5 for 24 h with 3 changes of dialysis buffer. The concentration of oxymyoglobin was determined by measuring the absorbance at 525 nm (Brown and Mebine, 1969).

Natural actomyosin (NAM) preparation

NAM was prepared according to the method of Benjakul *et al*. (1997). Bluefish muscle (10 g) was homogenized in a Waring blender with 100 ml chilled 0.6 M KCl, pH 7.0 (4°C) for 4 min. To avoid overheating during extraction, homogenization was performed in an ice bath. The homogenate was centrifuged at 5,000×*g* for 30 min at 4°C. The NAM pellet was resuspended and washed in 3 vol chilled distilled water. The final NAM preparation was collected by centrifuging at 5,000×*g* for 20 min at 4°C, and the pellet was dissolved by stirring for 30 min at 4°C in an equal volume of chilled 1.2 M KCl, pH 7.0. Undissolved material was removed from the preparation by centrifugation at 5,000×*g* for 20 min at 4°C. The protein concentration of NAM was determined using a BCA protein assay kit (Sigma, St. Louise, MO, USA) and diluted to 5 mg/ml.

Interaction between tuna myoglobin and NAM of fresh and frozen bluefish

NAM from fresh and frozen bluefish (whole and fillet stored at -20°C for a month) were prepared as previously described. To prepare myoglobin-NAM mixture model system, tuna oxymyoglobin (0.5 mg/ml) dissolved in 10 mM phosphate buffer, pH 6.5, containing 0.6 M KCl was mixed with NAM from either fresh or frozen bluefish (5 mg/ml) solubilized in the same buffer at the ratio of 1:1 (v/v). The oxidation of oxymyoglobin was determined after incubation at 4°C for 24 h. To remove the unbound myoglobin, 3 volumes of cold 10 mM phosphate buffer, pH 6.5 was added to the solution, mixed thoroughly, and centrifuged at 5,000×*g* for 30 min at 4°C. Whiteness, Ca^{2+}-ATPase activity and protein patterns of the pellet were determined. Myoglobin concentration in the supernatant was measured and the percentage of

bound myoglobin content was then calculated via the difference between total myoglobin content added and that found in the supernatant.

Effect of aldehydes on interaction between tuna myoglobin and bluefish NAM

Tuna oxymyoglobin (0.5 mg/ml) dissolved in 10 mM phosphate buffer, pH 6.5, containing 0.6 M KCl was mixed with bluefish NAM (5 mg/ml) solubilized in the same buffer at the ratio of 1:1 (v/v). The mixture was then added with hexanal or hexenal (0.1 mM in ethanol) at the ratio of 1:1 (v/v). Controls were aldehyde-free, but contained an equal volume of ethanol. After incubation at 4°C for 24 h, unbound aldehydes were removed by dialysis against 10 vol of 10 mM phosphate buffer, pH 6.5, containing 0.6 M KCl at 4°C and the oxidation of oxymyoglobin in the reaction mixture was determined. To remove the unbound myoglobin, 3 vol of cold 10 mM phosphate buffer, pH 6.5 was added to the solution, mixed thoroughly, and centrifuged at 5,000×*g* for 30 min at 4°C. Whiteness, Ca^{2+}-ATPase activity and protein patterns of the pellet were determined. Percentage of bound myoglobin was then calculated.

Determination of oxymyoglobin oxidation

The oxidation of oxymyoglobin during incubation in the presence or the absence of NAM was determined spectrophotometrically by scanning from 650 to 450 nm with a diffuse-integrating sphere attached to a Shimadzu UV-2100 spectrophotometer. The ratio of A_{630} to A_{525} was calculated according to Hansen and Sereika (1969). A high A_{630}/A_{525} ratio indicates a high relative proportion of metmyoglobin.

Protein patterns by SDS-PAGE

The protein patterns of myoglobin, NAM, and washed NAM-myoglobin mixture were visualized by SDS-PAGE using a 4% stacking gel and 15% separating gel according to Laemmli (1970). The current for each gel was maintained at 10 mA using a Mini Protean II unit

(Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.1% (w/v) Coomassie brilliant blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid, and destained with 40% methanol (v/v) and 7% (v/v) acetic acid. A protein standard containing myosin (206 kDa), □-galactosidase (124 kDa), bovine serum albumin (83 kDa), carbonic anhydrase (42.3 kDa), soybean trypsin inhibitor (32.2 kDa), lysozyme (18.8 kDa) and aprotinin (7 kDa) was used. The densitometric analysis of the protein bands was carried out using the Quantity One, Quantitation Software Version 4.1 (Bio-Rad Laboratories, Inc., Richmond, CA, USA).

Determination of Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activity of NAM and myoglobin-NAM mixture was determined according to Benjakul *et al*. (1997). NAM in model systems was diluted to 2.5 to 8 mg/ml with 0.6 M KCl, pH 7.0. Diluted NAM solution (1 ml) was added to 0.6 ml of 0.5 M Tris-maleate, pH 7.0. The mixture was combined with 1 ml of 0.1 M $CaCl_2$ and the deionized water was added to a total volume of 9.5 ml. One-half ml of 20 mM adenosine 5'-triphosphate (ATP) solution was then added to initiate the assay reaction, which was conducted for 8 min at 25°C and terminated by adding 5 ml of chilled 15% (w/v) trichloroacetic acid. The reaction mixture was centrifuged at 3,500×*g* for 5 min and the inorganic phosphate content of the supernatant was measured by the method of Fiske and Subbarow (1925). Ca^{2+}-ATPase activity was expressed as μmoles inorganic phosphate released/mg NAM protein/min. A blank solution was prepared by adding the chilled trichloroacetic acid prior to addition of ATP.

Determination of whiteness

Colorimetric values of the NAM and washed NAM-myoglobin mixture were obtained by using a Minolta Chromameter (Model CR-200b, Osaka, Japan). L* (lightness), a* (redness/greenness) and b* (yellowness/blueness) were measured and the whiteness was calculated as described by Park (1994):

$$\text{Whiteness} = 100-\left[(100-L^*)^2+a^{*2}+b^{*2}\right]^{1/2}.$$

Statistical analysis

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 10.0 for windows, SPSS Inc., Chicago, USA).

9.4 Results and Discussion

Interaction between tuna myoglobin and NAM extracted from fresh and frozen bluefish

Higher bound myoglobin content was observed in NAM-myoglobin mixture, in which NAM was extracted from both frozen fillet and whole bluefish, compared with the mixture containing NAM extracted from fresh bluefish (Figure 46).

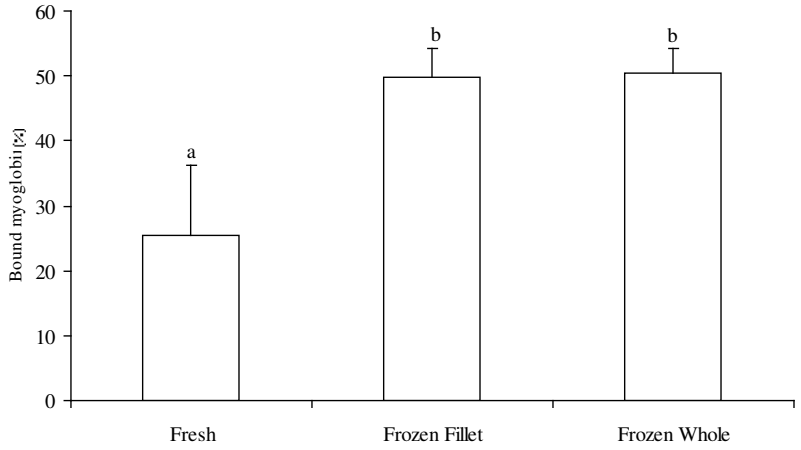


Figure 46. Bound myoglobin content (%) in the mixture of tuna myoglobin-NAM from fresh and frozen bluefish incubated at 4°C for 24 h. Bars represent the standard deviation from ten determinations. Different letters indicate significant differences (P<0.05).

The result suggested that NAM extracted from frozen fish bound to myoglobin comparatively higher than did NAM extracted from fresh fish. However, no differences in bound myoglobin content in myoglobin-NAM prepared from frozen whole fish and from fillet were found (P>0.05). The bound myoglobin content observed in NAM prepared from fresh fish was 25.44%, whereas 49.78 and 50.44% bound myoglobin were found in the mixture comprising NAM prepared from frozen fillet and frozen whole fish, respectively. Frozen storage caused the denaturation or unfolding of myofibrillar proteins. As the freezing process continues, the hydrophobic as well as the hydrophobic regions of the protein molecules become exposed to a new environment, which may allow the formation of intermolecular cross-linkages (Lanier, 2000; Sikorski and Kolakowska, 1994). Additionally, reactive groups of unfolded myofibrillar proteins could further interact with myoglobin, resulting in the increased bound myoglobin in washed NAM-myoglobin mixture. Thus, attachment of myoglobin to denatured myofibrillar proteins occurred easily, leading to the retention of myoglobin that would otherwise be removed through the washing process. Therefore, the NAM extracted from frozen fish seemed to adduct with myoglobin easier than that extracted from fresh fish.

The effect of freeze-induced myofibrillar proteins changes on the formation of metmyoglobin in NAM-myoglobin mixture is depicted in Figure 47. Metmyoglobin content in the control myoglobin was generally lower than that of myoglobin in the presence of NAM. The addition of NAM into myoglobin solution most likely enhanced the adduction between these proteins. During the interaction, globin possibly underwent conformation changes, resulting in the instability of myoglobin. From the result, metmyoglobin formation in the mixture of myoglobin-NAM extracted from fresh and frozen fillet was not different (P>0.05), suggesting that the binding of myoglobin to NAMs might occur at the similar extent. However, the oxidation of myoglobin in the presence of NAM prepared from the frozen whole fish increased markedly (P <0.05; Figure 47).

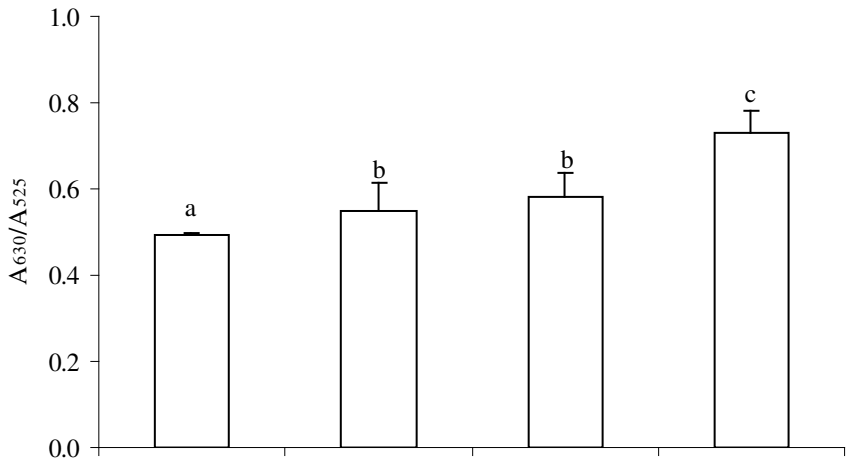


Figure 47. Metmyoglobin formation (A_{630}/A_{525}) of tuna myoglobin and the mixture of tuna myoglobin-NAM from fresh and frozen bluefish incubated at 4°C for 24 h. Bars represent the standard deviation from ten determinations. Different letters indicate significant differences ($P<0.05$).

Muscle tissues of frozen whole fish possibly underwent the greater chemical and/or biochemical changes than did the tissues of frozen fillet and fresh fish. Whole fish contained viscera, blood and belly lipid and they were rich in enzymes, prooxidants and substrates for chemical and/or biochemical reactions. These components likely involved in the muscle denaturation as well as lipid oxidation. Among the reaction products formed, lipid oxidation products might affect the NAM extracted by inducing the conformational changes as well as binding tightly to NAM. When the NAM reacted with myoglobin in a model system, the oxidation of myoglobin might be pronounced, caused by NAM itself as well as lipid oxidation products bound to NAM.

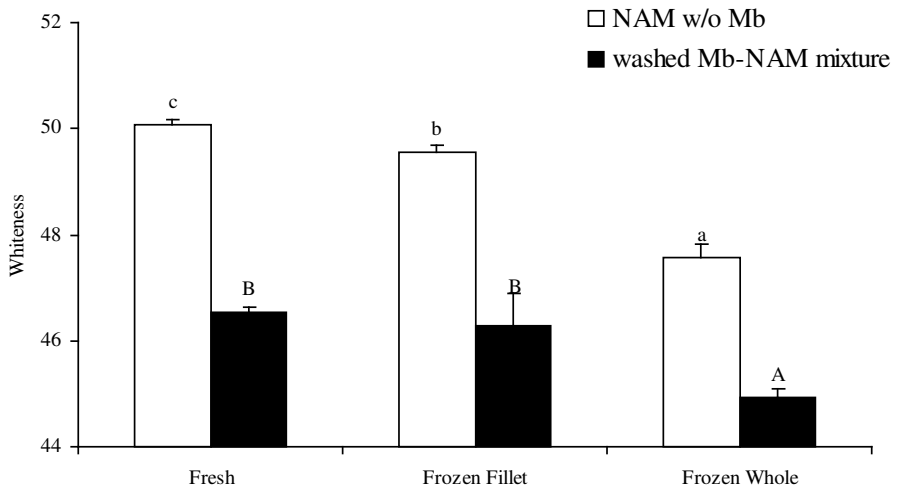


Figure 48. Whiteness values for washed bluefish NAM (□) and washed tuna myoglobin-NAM from fresh and frozen bluefish (□) incubated at 4°C for 24 h. Bars represent the standard deviation from ten determinations. Different letters or different letter case indicates significant differences (P<0.05).

The whiteness of NAM extracted from frozen fillet was lower than that of NAM from fresh fish (P<0.05; Figure 48). The decrease in whiteness was more pronounced in NAM extracted from frozen whole fish, compared to that extracted from frozen fillet. The discoloration of NAM extracted from frozen fish was possibly due to the marked aggregation of muscle proteins. The rupturing of different bonds in the native conformation of proteins in frozen fish is followed by side-by-side aggregation of myofibrillar proteins (Connell, 1960). Furthermore, whole fish might undergo the lipid oxidation, in which aldehydes or carbonyl compounds were produced. Those compounds might interact with amino group via Maillard reaction and the reaction products, which are brown in color, might lower the whiteness of NAM extracted. The presence of myoglobin in the NAM solution resulted in the decreased whiteness of washed myoglobin-NAM mixture (P<0.05; Figure 48). This result indicated that some myoglobin bound to NAM to some extent, leading to the decrease in whiteness. NAM extracted from frozen fish, especially whole fish, had the marked decrease in whiteness when myoglobin was combined. However, no differences in whiteness between washed myoglobin-NAM mixture prepared from fresh fish and frozen fillet were found (P>0.05; Figure 48). The lower whiteness of washed myoglobin-NAM mixture was in accordance with the increase in metmyoglobin formed in the mixture (Figure 47). As a consequence, the interaction between NAM and myoglobin was positively associated with the formation of metmyoglobin.

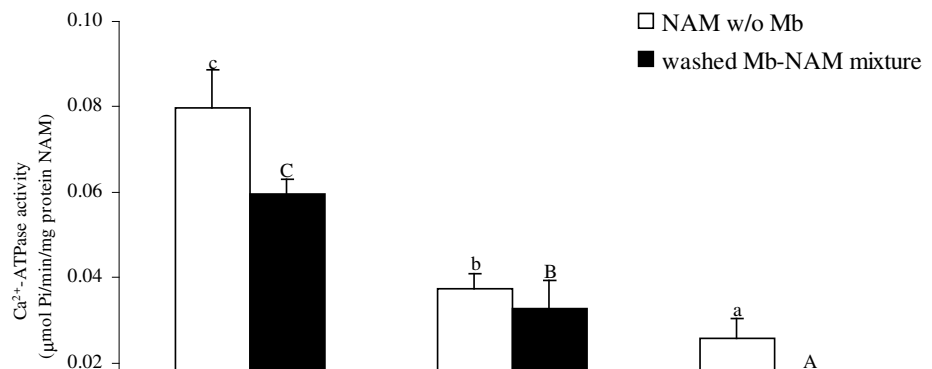


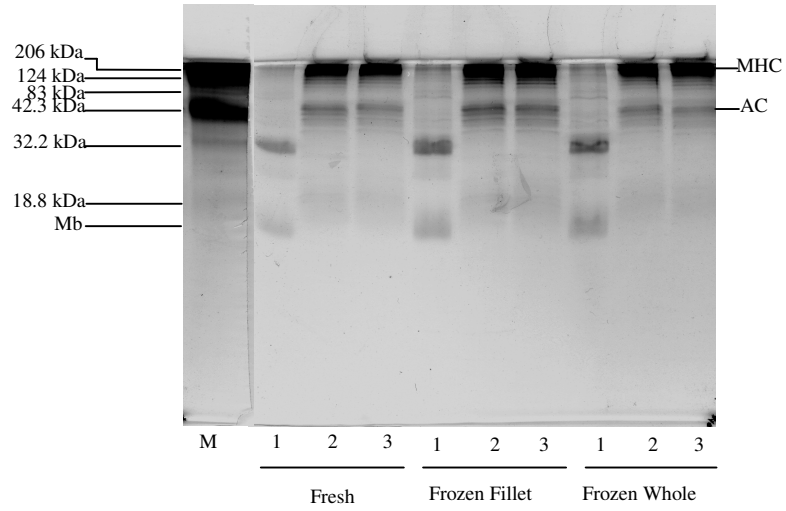
Figure 49. Ca^{2+}-ATPase activity of bluefish NAM (□) and tuna myoglobin-NAM from fresh and frozen bluefish (□) incubated at 4°C for 24 h. Bars indicate standard deviation from ten determinations. Different letters or different letter case indicates significant differences (P<0.05).

In the absence of myoglobin, Ca^{2+}-ATPase activity of NAM extracted from frozen fish was lower than that extracted from fresh fish (P<0.05; Figure 49). The Ca^{2+}-ATPase activity of NAM extracted from frozen fillet and frozen whole fish decreased by 50% and 62.5%, respectively, when compared to that of NAM extracted from fresh fish. Jiang and Lee (1985) reported that the actomyosin Ca^{2+}-ATPase activity can be used as an index of protein quality during frozen storage. The loss of enzymatic activity reflects the extent of freeze damage and alteration of the protein structure in the muscle system (Jiang and Lee, 1985). The decrease in Ca^{2+}-ATPase activity of NAM was possibly due to the denaturation and/or aggregation of NAM during frozen storage. From the result, the changes in myosin of frozen whole fish took place at the highest extent as indicated by the lowest Ca^{2+}-ATPase activity. In the presence of myoglobin, Ca^{2+}-ATPase activity of myoglobin-NAM mixture was lower than that of NAM extracted from fresh fish (P<0.05). The interaction between myoglobin and NAM might induce the conformational change of myosin. Additionally, the binding of myoglobin possibly occurred at the head portion, which possesses the Ca^{2+}-ATPase activity. However, the highest activity remained was found in the mixture of myglobin-NAM extracted from fresh fish (P<0.05),

compared with myoglobin-NAM extracted from frozen fillet or whole fish. Among all mixtures, the lowest Ca^{2+}-ATPase activity was found in washed myoglobin-NAM prepared from frozen whole fish ($P<0.05$). From the result, it can be inferred that binding of myoglobin to NAM caused the decrease in Ca^{2+}-ATPase activity, regardless of the conformation of NAM.

Intermolecular cross-linkages between myoglobin and NAM resulted in the formation of high-molecular-weight polymers appeared on top of the gels in both reducing and non-reducing conditions (Figure 50A and 50B). A decrease in density of MHC and actin bands was observed with the concomitant disappearance of the myoglobin band (Figure 50A and 50B). However, similar protein patterns of the mixture of myoglobin-NAM extracted from fresh and frozen fish were observed (Figure 50A and 50B). From the result, some differences in protein patterns were observed between reducing and non-reducing conditions. Some actin band was regained under reducing condition (Figure 50B). This result suggested that myoglobin most likely bound with NAM extracted from both fresh and frozen fish via both disulfide and non-disulfide bond.

(A)



(B)

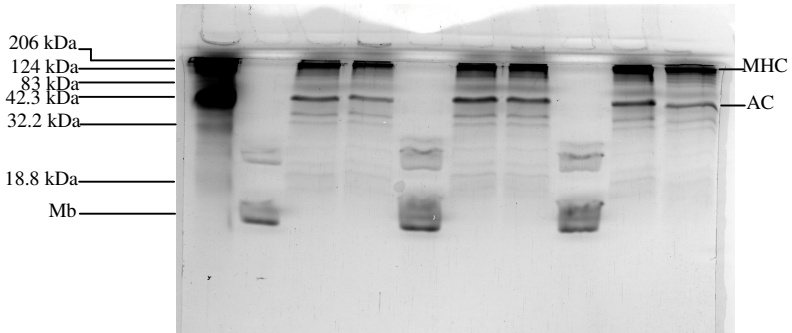


Figure 50. SDS-PAGE patterns of tuna myoglobin, washed bluefish NAM, and washed tuna myoglobin-NAM from fresh and frozen bluefish incubated at 4°C for 24h under non-reducing (A) and reducing conditions (B). M, molecular weight standards; 1, myoglobin; 2, washed NAM; 3, washed myoglobin-NAM mixture. MHC, myosin heavy chain; AC, actin. Myoglobin protein loaded onto SDS-PAGE was equal to the bound myoglobin content measured in the washed myoglobin-NAM mixture, while the amount of NAM protein loaded was equal to NAM measured in washed myoglobin-NAM mixture.

Effect of aldehydes on interaction between tuna myoglobin and bluefish NAM

The greater bound myoglobin content was found in the myoglobin-NAM mixture in the presence of aldehydes when compared with that of control (without aldehyde) (Figure 51). In the absence of aldehyde, the content of bound myoglobin was 23.68%, whereas bound myoglobin contents of 75.66 and 79.93% were found in the presence of hexanal and hexenal, respectively. The aldehydes added could act as the protein cross-linker between NAM and myoglobin. The modification of NAM and/or myoglobin by aldehyde could contribute to the increased binding of those proteins.

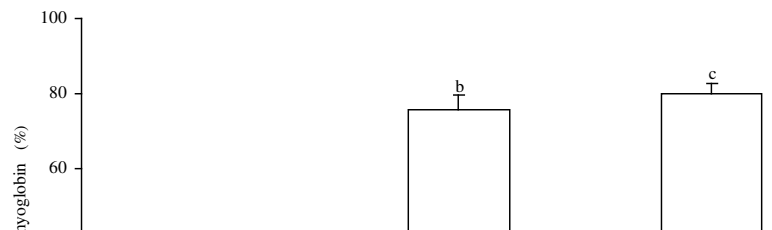


Figure 51. The effect of aldehydes on bound myoglobin content (%) in tuna myoglobin-bluefish NAM mixture incubated at 4°C for 24 h. Bars represent the standard deviation from ten determinations. Different letters indicate significant differences ($P<0.05$).

From the result, the bound myoglobin content was higher in the presence of hexenal, unsaturated aldehyde, than that found in the presence of saturated counterpart of equivalent carbon chain length (hexanal). Saturated and unsaturated aldehydes appear to interact differently with functional groups in protein (Faustman and Wang, 2000). Kautiainen (1992) reported that saturated aldehydes tended to form Schiff base adducts with the protein, while □,□-unsaturated aldehydes formed mixtures of Schiff bases and Michael addition products. The degree of saturation might be the one of factors determined the cross-linking activity of these aldehydes. The higher bound myoglobin content observed with aldehyde-treated samples might be due to the structural changes in both myoglobin and NAM induced by covalent attachment of the aldehyde. The structural changes of both proteins could expose more reactive groups, which readily formed protein-protein interactions or aggregations. Low density lipoprotein (LDL) was modified by hexanal (Chen *et al*., 1992) or by malonaldehyde (Haberland *et al*., 1988). Modification of hemoglobin by pentanal, hexanal, hexenal, heptanal, octenal, and nonenal was reported by Kautiainen (1992). Myoglobin was modified by 4-hydroxynonenal (4-HNE) (Lynch and Faustman, 2000). Malondialdehyde from degradation of oxidized polyunsaturated fatty acids has been shown to induce the cross-links of myosin through Schiff's base reactions (Buttkus, 1967).

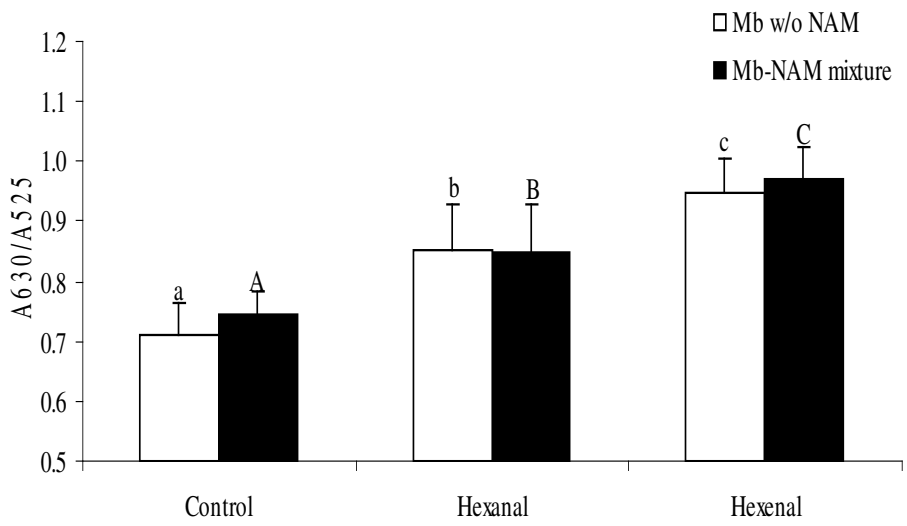


Figure 52. The effect of aldehydes on metmyoglobin formation (A_{630}/A_{525}) of tuna myoglobin and tuna myoglobin-bluefish NAM mixture incubated at 4°C for 24 h. Bars represent the standard deviation from ten determinations. Different letters indicate significant differences (P<0.05).

The effects of aldehydes on the redox stability of myoglobin and NAM-myoglobin mixture are depicted in Figure 52. During 24 h incubation, the metmyoglobin formation in the presence of aldehydes was higher than that observed in the absence of aldehydes in both myoglobin and myoglobin-NAM mixture (Figure 52). Lynch and Faustman (2000) reported that the covalent attachment of aldehyde to oxymyoglobin caused oxymyoglobin more susceptible to oxidation. The addition of hexenal to myoglobin and myoglobin-NAM mixture resulted in the higher oxymyoglobin oxidation than did the addition of hexanal (P<0.05). Of equivalent carbon chain length, unsaturated aldehyde demonstrated greater prooxidant activity toward myoglobin than the saturated counterparts (Faustman *et al*., 1999). From the result, aldehydes played an essential role in the oxidation of myoglobin regardless of the presence of NAM.

The whiteness of NAM and myoglobin-NAM mixture with and without aldehyde are present in Figure 53. The whiteness of both NAM and myoglobin-NAM mixture markedly decreased in the presence of aldehydes (P<0.05).

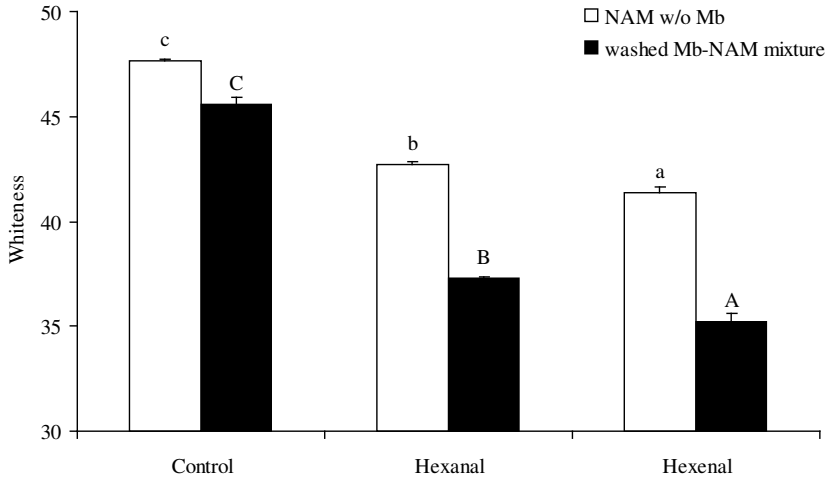


Figure 53. The effect of aldehydes on whiteness values for washed bluefish NAM (□) and washed tuna myoglobin-bluefish NAM mixtures (□) incubated at 4°C for 24 h. Bars represent the standard deviation from ten determinations. Different letters or different letter case indicates significant differences (P<0.05).

Generally, hexenal showed the pronounced effect on the discoloration of NAM and NAM-myoglobin than did hexanal (P<0.05). The result was in accordance with the bound myoglobin content which was greater in the presence of aldehyde (Figure 51). Without myoglobin, whiteness of NAM also decreased when the aldehyde was incorporated (P<0.05). The aldehyde added could induce the aggregation of NAM, resulting in the decreased whiteness of NAM. Li and King (1999) reported that malonaldehyde, a byproduct of lipid oxidation, caused the more hydrophobic exposure, loss of □-helix structure in the protein and further reacted with specific amino acids, such as free amino. As a consequence, the interaction between myoglobin and NAM occurred progressively in the presence of aldehydes, resulting in the decreased whiteness of washed myoglobin-NAM mixture. From the result, the considerable decrease in whiteness of washed myoglobin-NAM when both myoglobin and aldehydes were included indicated that myoglobin could bind tightly to NAM using aldehydes as the effective cross-linkers. As a result, the removal of myoglobin bound to NAM was markedly decreased.

The Ca^{2+}-ATPase activity of NAM and myoglobin-NAM mixture with and without aldehydes are depicted in Figure 54. The highest activity of Ca^{2+}-ATPase was found in NAM without myoglobin and aldehydes. The activity considerably decreased when the aldehyde was introduced (P<0.05). Without myoglobin in the mixture, hexenal showed the greater impact on the decrease in Ca^{2+}-ATPase activity than did hexanal. However, no differences in Ca^{2+}-ATPase activity between hexanal and hexenal treated samples were observed when myoglobin was combined with NAM (P>0.05). The presence of aldehydes during incubation possibly caused the destruction of the active sites of the Ca^{2+}-ATPase in the globular heads of myosin. Li and King (1999) reported that the decreased Ca^{2+}-ATPase activity during incubation with malonaldehyde was possibly owing to the conformation changes and the cross-linking of the proteins via formation of covalent bonds.

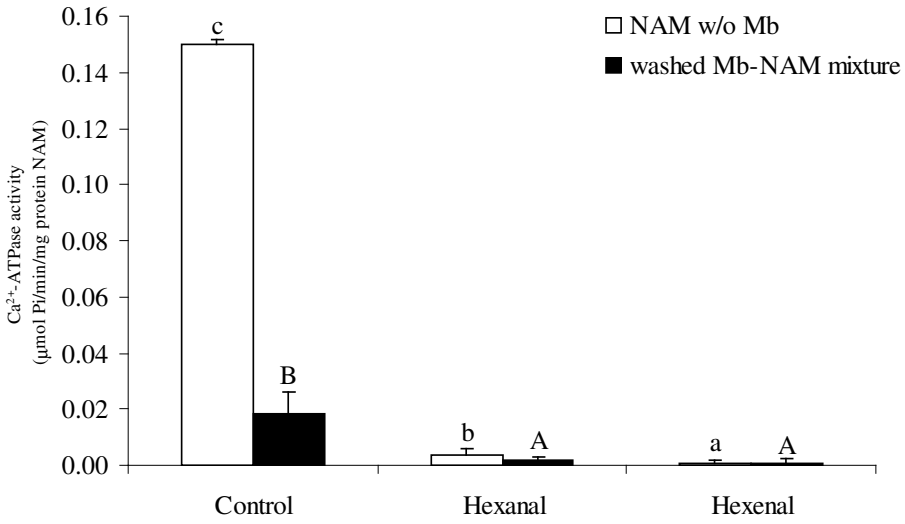


Figure 54. The effect of aldehydes on Ca^{2+}-ATPase activity of bluefish NAM (□) and tuna myoglobin-bluefish NAM mixture (□) incubated at 4°C for 24 h. Bars indicate standard deviation from ten determinations. Different letters or different letter case indicates significant differences (P<0.05).

MHC and actin band intensities decreased markedly when aldehydes were added (Figure 55A and 55B). Under reducing condition, it was found that some MHC as well as actin were regained for the NAM and myoglobin-NAM containing no aldehydes and hexanal. Nevertheless, only small protein bands were recovered for NAM or myoglobin-NAM treated with hexenal. The result suggested that the interaction of NAM or NAM and myoglobin might govern by disulfide bond in the presence of hexanal. However, disulfide bond showed the negligible effect on cross-linking induced by hexenal.

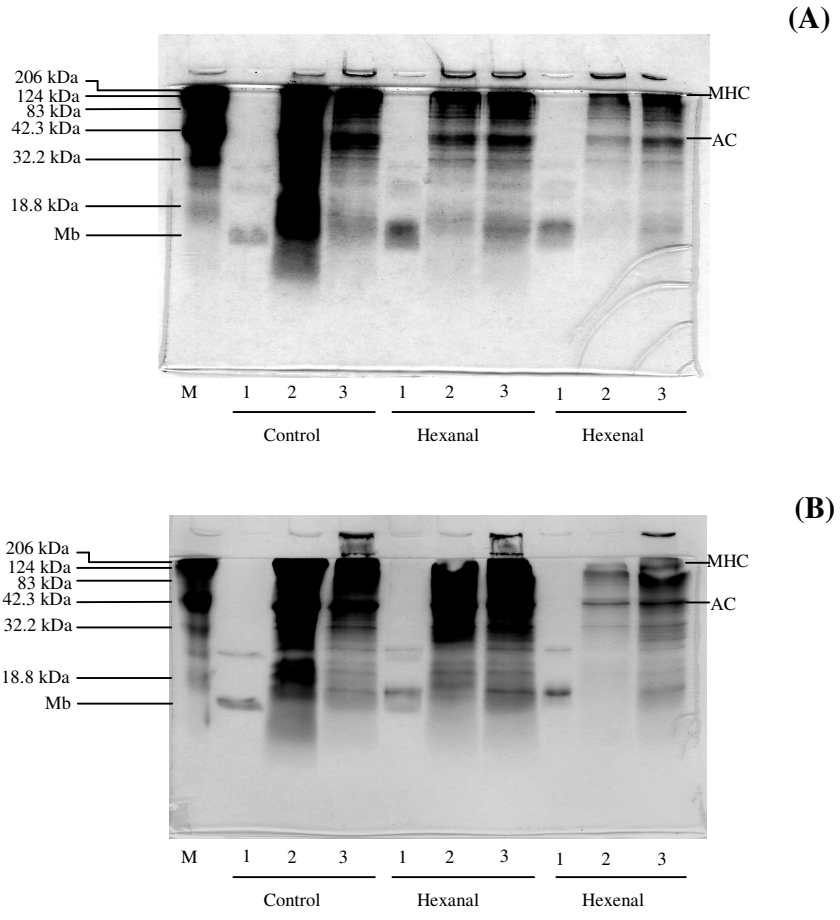


Figure 55. SDS-PAGE patterns of tuna myoglobin, washed bluefish NAM, and washed tuna myoglobin-bluefish NAM mixture incubated at 4°C for 24 h as affected by aldehydes during under non-reducing (A) and reducing conditions (B). M, molecular weight standards; 1, myoglobin; 2, washed NAM; 3, washed myoglobin-NAM

mixture. MHC, myosin heavy chain; AC, actin. Myoglobin protein loaded onto SDS-PAGE was equal to the bound myoglobin content measured in the washed myoglobin-NAM mixture.

9.5 Conclusions

In conclusion, the changes in myofibrillar proteins during frozen storage induced the binding of myoglobin and NAM during incubation at 4°C for 24 h. The binding of myoglobin to myofibrillar proteins decreased the activity of Ca^{2+}-ATPase with the coincidental formation of high-molecular-weight aggregate and resulted in the lowered whiteness of washed myoglobin-NAM mixture. Aldehyde lipid oxidation products exhibited the cross-linking activity toward myoglobin-NAM mixture and increased metmyoglobin formation. Aldehydes induced the binding between myoglobin and NAM, leading to the decrease in whiteness of washed myoglobin-NAM mixture. The binding was more pronounced in the presence of aldehydes especially unsaturated aldehyde. Therefore, the extended *post-mortem* handling or storage of fish raw materials may be associated with the increased binding between heme proteins and myofibrillar protein, leading to greater discoloration of processed fish muscle.