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The Effect of Freezing and Aldehydes on the Interaction between Fish Myoglobin and Myofibrillar Proteins

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The interaction between fish myoglobin (Mb) and natural actomyosin (NAM) extracted from fresh and frozen fish was studied. The quantity of soluble Mb in Mb−NAM extracted was less in frozen than in fresh fish ($P < 0.05$). However, no differences were observed in Mb that remained in solution following preparation of Mb−NAM from frozen whole fish vs frozen fillets ($P > 0.05$). MetMb formation in Mb−NAM was generally greater than that observed in control Mb ($P < 0.05$); the greatest MetMb content occurred in Mb−NAM extracted from frozen whole fish ($P < 0.05$). The effect of different aldehyde oxidation products on the interaction between fish Mb and NAM was also studied in vitro. The loss of soluble Mb from NAM:Mb preparations was greater in the presence of hexenal and hexanal ($P < 0.05$) relative to controls, and the degree of solubility loss varied with aldehyde type. Hexenal caused greater OxyMb oxidation than hexanal ($P < 0.05$). Whiteness of washed NAM and NAM−Mb mixtures decreased following aldehyde addition ($P < 0.05$). In the absence of Mb, the Ca^{2+}-ATPase activity of NAM was lower with added hexenal than with hexanal ($P < 0.05$). However, no differences in Ca^{2+}-ATPase activity between hexanal and hexenal-treated samples were observed when Mb was present ($P > 0.05$). Reducing and nonreducing sodium dodecyl sulfate−polyacrylamide gel electrophoresis analyses suggested that both disulfide and nondisulfide covalent linkages contributed to aldehyde-induced cross-linking between Mb and myofibrillar proteins.

KEYWORDS: Fish myoglobin; myofibrillar proteins; interaction; aldehyde; frozen

INTRODUCTION

Residual myoglobin (Mb) and hemoglobin influence the degree of whiteness of washed fish mince, a critical factor determining surimi gel quality (*1*). In general, hemoglobin is lost readily during handling and storage, whereas Mb is retained by the intracellular structure (*2*). Normally, both heme proteins can be removed during the washing process of fresh fish leading to increased whiteness of the raw material and resulting surimi. However, heme proteins become less soluble as fish muscle quality deteriorates (*3*). Denaturation of Mb and/or myofibrillar proteins that occurs before or during processing can enhance their interaction and cause discoloration of surimi (*4*). After capture, fish are normally kept in ice or frozen prior to unloading (*5*). During this stage, discoloration of muscle can occur and binding of pigments to muscle also takes place (*4*). Fish myofibrillar proteins are highly susceptible to physicochemical and structural modifications caused by freezing and frozen storage, and this leads to intramolecular conformational rearrangements and intermolecular aggregation (*6*). Benjakul and Bauer (*7*) reported that deterioration of frozen stored fish quality was due to the osmotic removal of water, denaturation of protein, and mechanical damage.

Apart from partial protein denaturation, lipid oxidation is a major problem in stored fish (*7*, *8*). Fatty fish may undergo rapid lipid oxidation during refrigerated storage due to their high content of polyunsaturated fatty acids (*9*). Chaijan et al. (*10*) 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 (*11*). Secondary products from lipid oxidation, especially aldehydes, can induce myofibrillar protein cross-linking, resulting in structural and functional changes in these proteins (*12*, *13*). Furthermore, aldehyde-based lipid oxidation products can alter Mb redox stability (*14*). Covalent modification of equine, bovine, porcine, and tuna Mbs by 4-hydroxynonenal, a product of linoleic acid

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oxidation, has been demonstrated (*15*−*18*). In addition, hexenal and hexanal were reported to accelerate the oxidation of tuna OxyMb (*18*).

The degradation and/or denaturation of muscle proteins during frozen storage and the formation of aldehyde lipid oxidation products during postmortem handling and storage may negatively affect the color of processed fish muscle. Therefore, the objectives of this study were to investigate the interaction between Mb and myofibrillar proteins extracted from fresh and frozen fish and to study the effect of aldehydes known to result from lipid oxidation, especially hexenal and its saturated counterpart, hexanal, on the interaction between Mb and myofibrillar proteins.

MATERIALS AND METHODS

Chemicals. Sephacryl S-200HR, sodium dodecyl sulfate (SDS), β-mercaptoethanol, Triton X-100, Tris(hydroxymethyl)aminomethane, sodium hydrosulfite, sodium phosphate, potassium chloride, and bicinchoninic acid (BCA) protein assay kit were obtained from Sigma (St. Louis, MO). Ammonium sulfate was purchased from Fisher Scientific (Fair Lawn, NJ).

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). 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 used for extraction of natural actomyosin (NAM). For the frozen samples, whole gutted fish and fillets were stored at −20 °C in an air-blast freezer; NAM was prepared from fish muscle after 1 month of storage. The longissimus muscles of yellowfin tuna were cut into 0.2 kg pieces and stored in vacuum packages at −20 °C until required for Mb isolation.

Extraction and Purification of Mb from Tuna Muscle. Extraction and purification of Mb were performed according to Trout and Gutzke (*19*) with a slight modification. 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 ethylenediaminetetraacetic acid (EDTA) and 25 g/L Triton X-100) for 1 min at high speed. The homogenate was centrifuged at 5000*g* for 10 min at 4 °C using a RC-5B centrifuge (Sorvall, Norwalk, CT); 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 obtain 65% saturation, and the suspension was stirred gradually for 60 min at 4 °C. The suspension was centrifuged at 18000*g* for 20 min at 4 °C, and the solid pellet was discarded. The supernatant was again 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 20000*g* for 60 min at 4 °C. The resulting soft Mb-containing pellet was dissolved in a minimal volume of cold 5 mM Tris-HCl buffer containing 1 mM EDTA, pH 8.0, which was referred to as the starting buffer. The mixture was then dialyzed against 10 vol of starting buffer for 24 h at 4 °C. The dialysate was immediately applied onto a Sephacryl S-200HR column (2.5 cm × 100 cm) previously equilibrated with starting buffer. The elution was conducted using starting buffer at a flow rate of 60 mL/h, and 5 mL fractions were collected.

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

Preparation of OxyMb. OxyMb was prepared by hydrosulfite-mediated reaction of purified tuna Mb (*20*), and the residual hydrosulfite was removed by dialyzing the sample against 10 vol of cold 10 mM phosphate buffer, pH 6.5, for 24 h. The concentration of OxyMb in the solution was determined by measuring the absorbance at 525 nm (*20*).

NAM Preparation. NAM was prepared according to the method of Benjakul et al. (*21*). Bluefish muscle (10 g) was homogenized in a Waring blender with 100 mL of 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 5000*g* for 30 min at 4 °C. The NAM pellet was resuspended and washed in 3 vol of chilled distilled water. The final NAM preparation was collected by centrifuging at 5000*g* for 20 min at 4 °C, and the resulting pellet was dissolved by stirring for 30 min at 4 °C in an equal vol of chilled 1.2 M KCl, pH 7.0. Undissolved material was removed from the preparation by centrifugation at 5000*g* for 20 min at 4 °C. The protein concentration of NAM was determined using a BCA protein assay kit (Sigma) and diluted to 5 mg/mL.

Interaction between Tuna Mb and NAM of Fresh and Frozen Bluefish. The design of this procedure measures loss of Mb from the supernatant obtained after NAM and Mb are combined and treated. The loss of soluble Mb in the supernatant could result from binding of Mb to NAM proteins or insolubilization and precipitation along with additional Mb molecules and/or NAM under the conditions used. It is important to note that if the Mb did become insoluble, it would not be precluded from reacting with NAM proteins, for example, via hydrophobic interactions, and thus, it would be expected to remain with the NAM fraction. To prepare the Mb−NAM model system, tuna OxyMb (0.5 mg/mL) dissolved in 10 mM phosphate buffer, pH 6.5, and containing 0.6 M KCl was mixed with NAM from either fresh or frozen bluefish (5 mg/mL) as described above and solubilized in the same buffer at a ratio of 1:1 (v/v). OxyMb oxidation was determined after 24 h of incubation at 4 °C. To remove Mb that did not associate with NAM, 3 vol of cold 10 mM phosphate buffer, pH 6.5, was added to the solution, mixed thoroughly, and centrifuged at 5000*g* for 30 min at 4 °C. Whiteness, Ca^{2+}-ATPase activity, and SDS−polyacrylamide gel electrophoresis (PAGE) protein patterns of the pellet were analyzed. The Mb concentration in the supernatant was measured, and the percentage of Mb associated with the NAM fraction was then calculated via the difference between total Mb content added and that recovered from the supernatant.

Effect of Aldehydes on Interaction between Tuna Mb and Bluefish NAM. Tuna OxyMb (0.5 mg/mL) dissolved in 10 mM phosphate buffer containing 0.6 M KCl, pH 6.5, was mixed with bluefish NAM (5 mg/mL) solubilized in the same buffer at a ratio of 1:1 (v/v). Hexanal or hexenal (0.1 mM stock in ethanol) was then added to the mixture to achieve a concentration ratio of 1:1 (v/v) with Mb. Controls were aldehyde-free but contained an equal volume of ethanol needed to deliver the aldehyde. After 24 h of incubation at 4 °C, unbound aldehydes were removed by dialysis overnight against 10 vol of 10 mM phosphate containing 0.6 M KCl, pH 6.5 at 4 °C, and the extent of OxyMb oxidation was determined. To remove soluble Mb, 3 vol of cold 10 mM phosphate buffer, pH 6.5, was added to the solution, mixed thoroughly, and centrifuged at 5000*g* for 30 min at 4 °C. Whiteness, Ca^{2+}-ATPase activity, and protein patterns of the pellet were determined. The percentage of Mb associated with NAM fraction was calculated as described above.

Determination of OxyMb Oxidation. OxyMb oxidation was determined spectrophotometrically by scanning the entire mixture from 650 to 450 nm with a diffuse-integrating sphere attached to a Shimadzu UV-2100 spectrophotometer (Shimadzu Scientific Instruments Inc.). The ratio of A_{630} to A_{525} was calculated according to Hansen and Sereika (*22*). A large A_{630}/A_{525} ratio indicates a high relative proportion of MetMb.

Protein Patterns by SDS-PAGE. The protein patterns of NAM−Mb mixtures were visualized by SDS-PAGE using a 4% stacking gel and 15% separating gel according to Laemmli (*23*) using a Mini Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA). The current for each gel was maintained at 10 mA. 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 consisting of myosin (206 kDa), β-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 as a reference. The densitometric analysis of the protein bands was carried out using

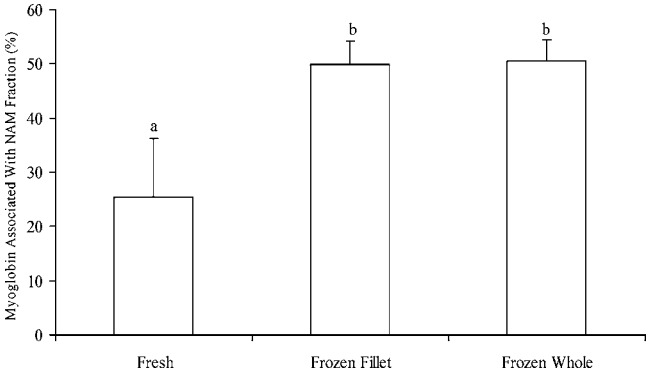

Figure 1. Mb content (%) that remained associated with the NAM fraction in tuna Mb–NAM mixtures from fresh and frozen bluefish incubated at 4 °C for 24 h. Bars represent the standard deviation from 10 determinations. Different letters indicate significant differences ($P < 0.05$).

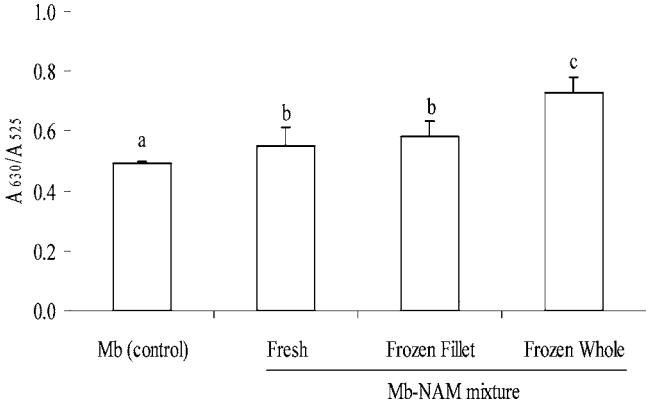

Figure 2. MetMb formation (A_{630}/A_{525}) of tuna Mb alone and in tuna Mb–NAM from fresh and frozen bluefish incubated at 4 °C for 24 h. Bars represent the standard deviation from 10 determinations. Different letters indicate significant differences ($P < 0.05$).

the densitometer programmed with Quantity One, Quantitation Software Version 4.1 (Bio-Rad Laboratories).

Determination of Ca^{2+}-ATPase Activity. The Ca^{2+}-ATPase activity of NAM and Mb−NAM mixtures was determined according to Benjakul et al. (*21*). NAM in model systems was diluted to 2.5−8 mg/mL with 0.6 M KCl, pH 7.0. The 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 deionized water was added to a total volume of 9.5 mL. One-half milliliter of 20 mM adenosine 5′-triphosphate (ATP) solution was then added to initiate the assay reaction and, following 8 min at 25 °C, was terminated by adding 5 mL of chilled 15% (w/v) trichloroacetic acid. The reaction mixture was centrifuged at 3500*g* for 5 min, and the inorganic phosphate content of the supernatant was measured (*24*). The Ca^{2+}-ATPase activity was expressed as μmol 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 pellet were obtained by using a Minolta Chromameter (model CR-200b, Osaka, Japan). L^* (lightness), a^* (redness/greenness), and b^* (yellowness/blueness) were measured; whiteness was calculated as described by Park (*25*):

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

Statistical Analysis. Data were subjected to analysis of variance. Comparison of means was carried out by Duncan's multiple range test (*26*). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 10.0 for windows, SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Interaction between Tuna Mb and NAM Extracted from Fresh and Frozen Bluefish. The model systems used were comprised of NAM extracted from fresh and frozen bluefish, which was then combined with buffer (control) or Mb. A greater loss of soluble Mb was measured in the NAM−Mb mixture in which NAM was extracted from frozen fillets and whole bluefish when compared with NAM extracted from fresh bluefish (**Figure 1**). However, there were no differences in the quantity of soluble Mb in Mb−NAM prepared from frozen whole fish vs frozen fillets ($P > 0.05$). The Mb content remaining with NAM prepared from fresh fish was 25.4%, whereas 49.8 and 50.4% Mb remained with the NAM fraction in frozen fillets and frozen whole fish, respectively. Frozen storage is known to cause partial denaturation or unfolding of fish myofibrillar proteins (*7*). During the freezing process, hydrophobic as well as hydrophilic regions of proteins become exposed and allow for the formation of intermolecular cross-links (*4*, *27*). Additionally, reactive groups of unfolded myofibrillar proteins could react with Mb in washed NAM−Mb mixtures. Thus, it is possible that attachment of Mb to denatured myofibrillar proteins from frozen fish led to the retention of Mb that would otherwise be removed through the washing process.

The effect of freeze-induced myofibrillar protein changes on the formation of MetMb in NAM−Mb mixtures is depicted in **Figure 2**. The MetMb content in the control Mb (i.e., no NAM present) preparation was generally less than that of Mb in the presence of NAM ($P < 0.05$). The addition of NAM and subsequent protein interaction could result in conformational changes in Mb, resulting in redox instability of OxyMb. MetMb formation in Mb−NAM mixtures extracted from fresh and frozen fillets was not different ($P > 0.05$). However, Mb oxidation in the presence of NAM prepared from frozen whole fish was greater than that from fresh and frozen fillets ($P < 0.05$; **Figure 2**). This suggests that muscle tissues of frozen whole fish underwent greater biochemical changes than did the tissues of fresh and frozen fillets. Whole fish contained viscera, blood, and belly lipid, which are rich in enzymes, pro-oxidants, and substrates known to affect postmortem quality (*7*). These components likely affected muscle protein denaturation as well as lipid oxidation (*7*). Pretreatment processing of fish, including heading and eviscerating prior to handling, retarded the deterioration presumably caused by proteolysis (*28*). Among the reaction products formed, reactive lipid oxidation products would be expected to affect NAM by inducing conformational changes prior to extraction. This could be expected to lead to greater protein:protein interactions between Mb and NAM, Mb insolubilization, and OxyMb oxidation.

The whiteness of NAM extracted from frozen fillets was less than that of NAM prepared from fresh fish ($P < 0.05$; **Figure 3**). The decreased whiteness was more pronounced in NAM extracted from frozen whole fish when compared to that extracted from frozen fillets. This is not surprising and is consistent with the effect of whole frozen fish NAM on OxyMb oxidation. The loss of native bonding integrity among amino acids in the native conformation of proteins in frozen fish caused the aggregation of myofibrillar proteins (*29*). Furthermore, whole fish containing belly lipid, subdermal fat layer, and viscera could be expected to more readily undergo lipid oxidation in which aldehydes or carbonyl compounds were produced. Those compounds would interact with protein amino groups via Maillard reactions, and any resulting colored reaction products would lower the whiteness of extracted NAM. The presence of Mb in the NAM solution resulted in decreased whiteness of washed Mb−NAM ($P < 0.05$; **Figure 3**). NAM extracted from

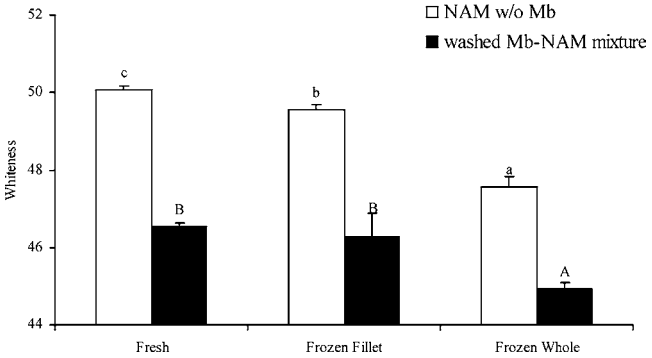

Figure 3. Whiteness values for washed bluefish NAM (□) and washed tuna Mb–NAM from fresh and frozen bluefish (■) incubated at 4 °C for 24 h. Bars represent the standard deviation from 10 determinations. Different letters or different letter cases indicate significant differences ($P < 0.05$).

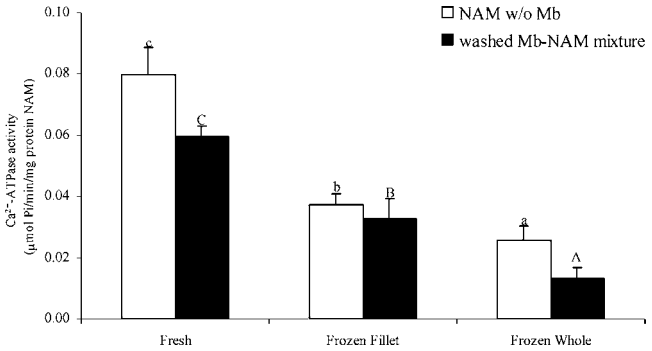

Figure 4. Ca^{2+}-ATPase activity of bluefish NAM (□) and tuna Mb–NAM from fresh and frozen bluefish (■) incubated at 4 °C for 24 h. Bars indicate standard deviation from 10 determinations. Different letters or different letter cases indicate significant differences ($P < 0.05$).

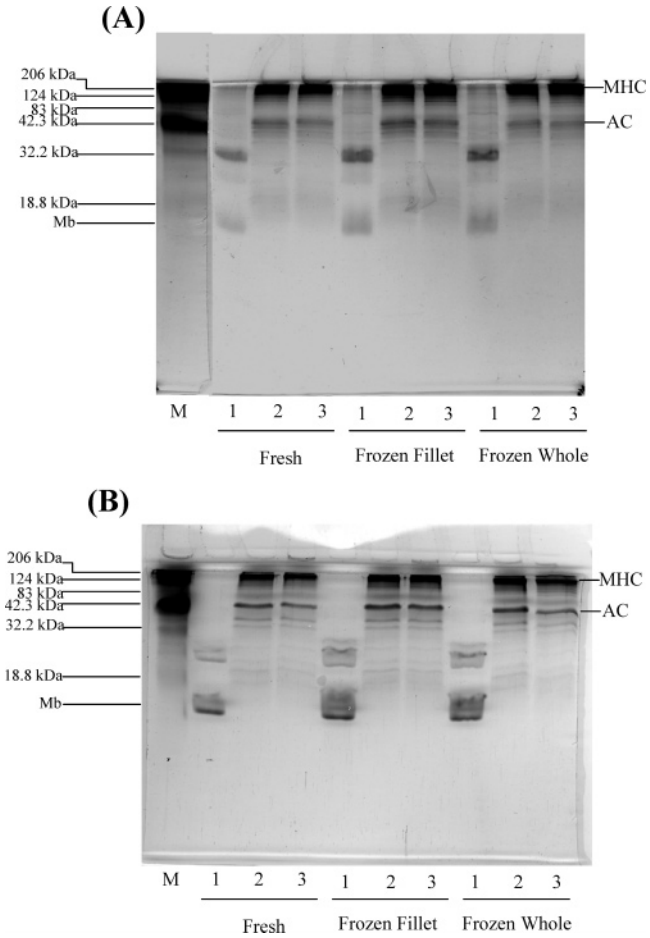

Figure 5. SDS-PAGE patterns of tuna Mb, washed bluefish NAM, and washed tuna Mb–NAM from fresh and frozen bluefish incubated at 4 °C for 24 h under nonreducing (**A**) and reducing conditions (**B**). M, molecular weight standards; 1, Mb; 2, washed NAM; 3, washed Mb–NAM mixture; and AC, actin. The Mb protein loaded onto SDS-PAGE was equal to the Mb associated with the NAM fraction and measured in the washed Mb–NAM mixture, while the amount of NAM protein loaded was equal to NAM measured in the washed Mb–NAM mixture.

frozen fish, especially whole fish, demonstrated a marked decrease in whiteness when Mb was included. However, no differences in whiteness between washed Mb−NAM mixtures prepared from fresh fillets and frozen fillets were found ($P > 0.05$; **Figure 3**). The lesser whiteness values of washed Mb−NAM mixtures were in accordance with observed increased MetMb (**Figure 2**). As a consequence, the interaction between NAM and Mb was associated positively with the formation of MetMb.

In the absence of Mb, the Ca^{2+}-ATPase activity of NAM extracted from frozen fish was less than that extracted from fresh fish ($P < 0.05$; **Figure 4**). The Ca^{2+}-ATPase activity of NAM extracted from frozen fillets and frozen whole fish decreased by 50 and 62.5%, respectively, when compared to that of NAM extracted from fresh fish. Jiang and Lee (*30*) reported that actomyosin Ca^{2+}-ATPase activity can be used as an index of fish protein quality during frozen storage. The loss of enzymatic activity reflects the extent of damage to, and alteration of, protein structure in the frozen muscle system (*30*). Decreased Ca^{2+}-ATPase activity of NAM could be attributed to denaturation and/or aggregation of myofibrillar proteins that occurred during frozen storage. In the presence of Mb, the Ca^{2+}-ATPase activity of fresh fish Mb−NAM was less than that of fresh fish NAM ($P < 0.05$). The interaction between Mb and NAM could alter myosin conformation, and if binding of Mb occurred at the myosin head, then significant losses of Ca^{2+}-ATPase activity would be expected. The greatest activity was measured in Mb−NAM extracted from fresh fish muscle ($P < 0.05$) as compared with Mb−NAM extracted from frozen fillet or frozen whole fish. Among all mixtures, the least Ca^{2+}-ATPase activity was recorded in washed Mb−NAM prepared from frozen whole fish ($P < 0.05$).

Intermolecular cross-linkages between Mb and NAM resulted in the formation of high molecular weight polymers, which were visualized at the top SDS gels prepared under both reducing and nonreducing conditions (**Figure 5A**,**B**). Decreased density of myosin heavy chain (MHC) and actin bands was observed with the concomitant disappearance of the Mb band (**Figure 5A**,**B**). However, protein patterns of Mb−NAM extracted from both fresh and frozen fish were similar (**Figure 5A**,**B**). Some differences in protein patterns between reducing and nonreducing conditions were observed ($P < 0.05$). For example, the actin band was more pronounced under reducing conditions ($P < 0.05$; **Figure 5B**).

Effect of Aldehydes on Interaction between Tuna Mb and Bluefish NAM. A greater Mb content associated with the NAM fraction was measured in Mb−NAM in the presence of aldehydes when compared with that of controls (without aldehyde) (**Figure 6**). In the absence of aldehyde, the Mb content associated with NAM was 23.7%, whereas 75.7 and 79.9% Mb remained with NAM fractions in the presence of hexanal and hexenal, respectively. The added aldehydes, specificially hexenal, could have acted as a protein cross-linker between NAM and Mb. The Mb content associated with the

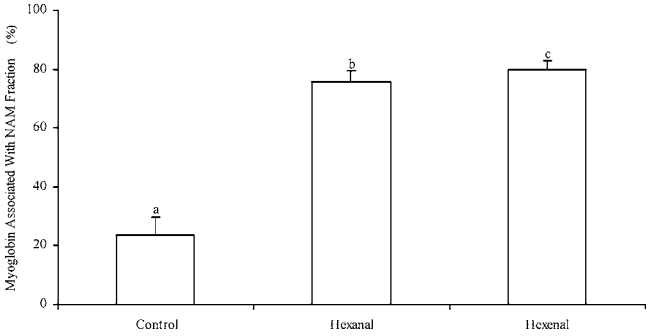

Figure 6. Effect of aldehydes on Mb content (%) associated with the NAM fraction in tuna Mb–bluefish NAM mixtures incubated at 4 °C for 24 h. Bars represent the standard deviation from 10 determinations. Different letters indicate significant differences ($P < 0.05$). NAM was extracted from fresh bluefish.

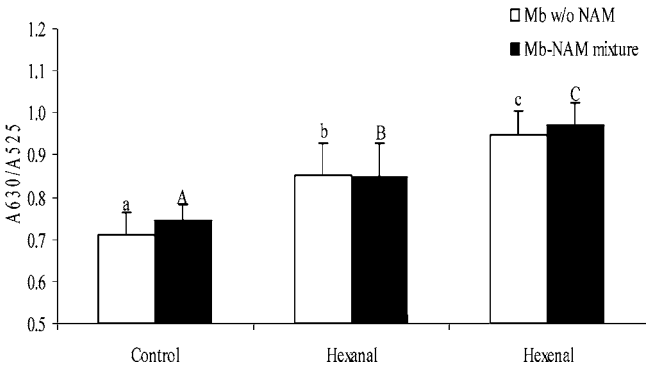

Figure 7. Effect of aldehydes on MetMb formation (A_{630}/A_{525}) of tuna Mb alone and tuna Mb–bluefish NAM mixtures incubated at 4 °C for 24 h. Bars represent the standard deviation from 10 determinations. Different letters indicate significant differences ($P < 0.05$). NAM was extracted from fresh bluefish.

NAM fraction was greater in the presence of hexenal than in its saturated counterpart of equivalent carbon chain length, hexanal ($P < 0.05$). Saturated and unsaturated aldehydes react differently with protein functional groups, and unsaturated aldehydes have at least two functional groups available for reaction with protein nucleophiles (*31*). Kautiainen (*32*) reported that saturated aldehydes tended to form Schiff base adducts, while α,β-unsaturated aldehydes (e.g., hexenal) formed mixtures of Schiff bases and Michael addition products. Malondialdehyde, formed from degradation of oxidized polyunsaturated fatty acids, has been shown to induce cross-links of myosin through Schiff's base reactions (*33*, *34*). The greater Mb content observed to be associated with NAM fractions in aldehyde-treated samples could result from structural changes in Mb and/or NAM induced by covalent modification of the aldehydes. Structural changes of proteins would be expected with aldehyde binding, and this could expose more reactive groups, leading to greater protein−protein interactions and aggregation.

The effects of aldehydes on the redox stability of Mb in control and NAM−Mb mixtures are depicted in **Figure 7**. During 24 h of incubation, MetMb formation was greater in the presence of aldehydes than in their absence for both Mb controls and Mb−NAM mixtures (**Figure 7**). Lynch and Faustman (*14*) reported that the covalent binding of aldehyde to OxyMb resulted in greater redox instability of OxyMb. The addition of hexenal to Mb controls and Mb−NAM mixtures caused greater OxyMb oxidation than did hexanal addition ($P < 0.05$).

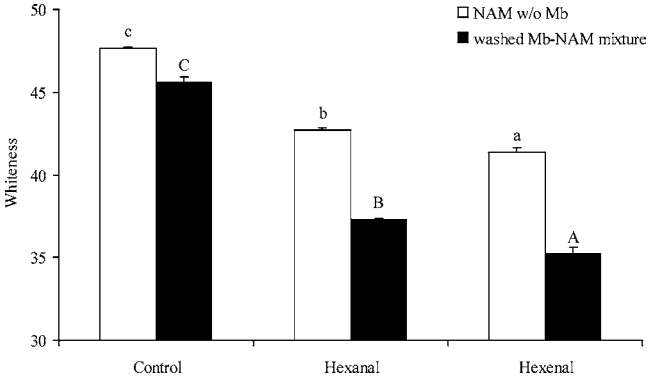

Figure 8. Effect of aldehydes on whiteness values for washed bluefish NAM (□) and washed tuna Mb–bluefish NAM mixtures (■) incubated at 4 °C for 24 h. Bars represent the standard deviation from 10 determinations. Different letters or different letter cases indicate significant differences ($P < 0.05$). NAM was extracted from fresh bluefish.

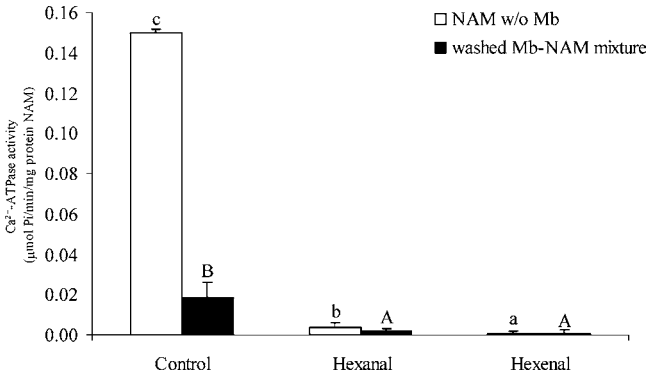

Figure 9. Effect of aldehydes on Ca^{2+}-ATPase activity of bluefish NAM (□) and tuna Mb–bluefish NAM mixtures (■) incubated at 4 °C for 24 h. Bars indicate standard deviation from 10 determinations. Different letters or different letter cases indicate significant differences ($P < 0.05$). NAM was extracted from fresh bluefish.

The whiteness of NAM controls and Mb−NAM mixtures, with and without aldehyde, is presented in **Figure 8**. Whiteness of both NAM controls and Mb−NAM mixtures was decreased in the presence of aldehydes ($P < 0.05$). This result was consistent with the greater amount of Mb observed to be associated with NAM fractions in the presence of aldehyde (**Figure 6**). Generally, hexenal treatment resulted in a more pronounced effect on discoloration of NAM and NAM−Mb than did hexanal ($P < 0.05$). Even in the absence of Mb, whiteness of NAM decreased when aldehyde was incorporated ($P < 0.05$). If aldehydes induced aggregation of NAM, then decreased whiteness of NAM would be expected. The enhanced interaction between Mb and NAM in the presence of aldehydes resulted in decreased whiteness of washed Mb−NAM mixtures.

Ca^{2+}-ATPase activities of NAM controls and Mb−NAM mixtures, with and without aldehydes, are presented in **Figure 9**. The greatest Ca^{2+}-ATPase activity was observed in NAM without Mb and aldehydes and decreased considerably when aldehydes were introduced ($P < 0.05$). In the absence of Mb, hexenal had a greater impact on decreasing Ca^{2+}-ATPase activity than did hexanal ($P < 0.05$). However, no differences in Ca^{2+}-ATPase activity between hexanal and hexenal-treated samples were observed when Mb was present ($P > 0.05$). The addition of aldehydes likely compromised the active sites of Ca^{2+}-ATPase in the globular heads of myosin. Li and King (*13*) reported that decreased Ca^{2+}-ATPase activity occurred following

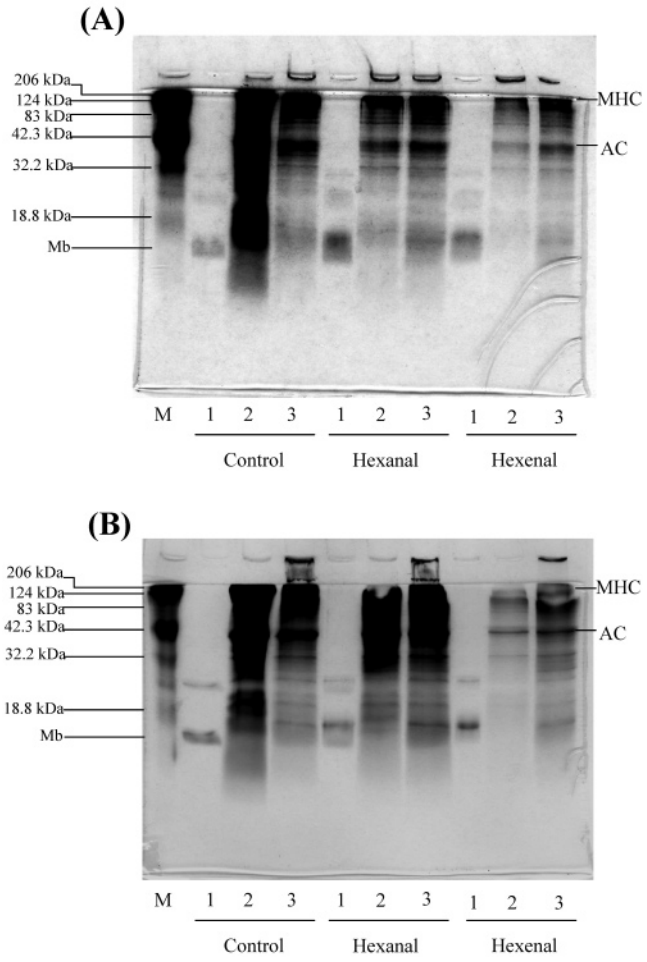

Figure 10. SDS-PAGE patterns of tuna Mb, washed bluefish NAM, and washed tuna Mb–bluefish NAM mixtures incubated at 4 °C for 24 h as affected by aldehydes during under nonreducing (**A**) and reducing conditions (**B**). M, molecular weight standards; 1, Mb; 2, washed NAM; 3, washed Mb–NAM mixture; and AC, actin. The Mb protein loaded onto SDS-PAGE was equal to the Mb associated with NAM faction and measured in the washed Mb–NAM mixture, while the amount of NAM protein loaded was equal to NAM measured in washed Mb–NAM mixture. NAM was extracted from fresh bluefish.

incubation with malondehyde and was likely due to conformational changes and covalent cross-linking of myosin.

MHC and actin band intensities decreased markedly when aldehydes were added to Mb−NAM mixtures ($P < 0.05$; **Figure 10A**,**B**). Under reducing conditions, the SDS-PAGE pattern revealed that some MHC and actin band intensities were recovered for NAM controls and Mb−NAM containing hexanal. However, protein band densities were considerably less intense only for NAM or Mb−NAM treated with hexenal.

Our results suggest that changes in myofibrillar proteins resulting from frozen storage of whole fish facilitated binding of Mb with NAM during incubation at 4 °C for 24 h. This decreased Ca^{2+}-ATPase activity led to formation of high molecular weight aggregates and resulted in lowered whiteness of washed Mb−NAM mixtures. Aldehyde lipid oxidation products favored cross-linking of Mb with myofibrillar proteins in Mb−NAM mixtures, increased MetMb formation, insolubilization of Mb (with or without subsequent interaction with NAM), and decreased whiteness of washed Mb−NAM mixtures. The effect was more pronounced in the presence of hexenal than its saturated counterpart, hexanal. Therefore, extended postmortem handling or storage of fish raw materials, which leads to enhanced lipid oxidation, may be associated with increased binding between heme and myofibrillar proteins leading to greater discoloration of the subsequently processed fish muscle.

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