CHAPTER 7

EFFECT OF IONIC STRENGTH AND TEMPERATURE ON INTERACTION

BETWEEN FISH MYOGLOBIN AND MYOFIBRILLAR PROTEINS

7.1 Abstract

The effects of ionic strength (0, 0.3 and 0.6 M KCl) and temperature (4 and 25°C) on the interaction between fish myoglobin and myofibrillar proteins were investigated in a model system. Increases in the relative content of bound myoglobin and metmyoglobin formation in myoglobin-natural actomyosin mixtures with concurrent decreases in whiteness and Ca^{2+}-ATPase activity were observed with increasing ionic strength ($P<0.05$). The relative content of bound myoglobin and the oxidation of oxymyoglobin were generally greater at 25°C than at 4°C ($P<0.05$). Binding of myoglobin to natural actomyosin (NAM) resulted in decreased whiteness ($P<0.05$). Ca^{2+}-ATPase was not affected by temperature ($P>0.05$). SDS-PAGE patterns of protein samples suggested that myoglobin-NAM interactions did not involve disulfide bonds. The formation of high-molecular-weight aggregates (>206 kDa) was observed and was more pronounced at higher ionic strength and higher temperature.

7.2 Introduction

Myoglobin is a globular heme protein found in the muscle of meat-producing animals (Faustman and Phillips, 2001). It has been known to be a major contributor to the color of muscle, depending upon its redox state and concentration. Myoglobin concentration is affected by both genetics and environment (Giddings, 1974; Livingston and Brown, 1981; Faustman *et al*., 1996). Color changes in meat can result from reaction of myoglobin with other muscle components, especially myofibrillar proteins (Hanan and Shaklai, 1995). The primary structures of fish myoglobins differ considerably from those of mammals (Brown, 1961). Haard (1992)

reported that yellowfin tuna myoglobin (146 residues) showed 79 to 85 amino acid substitutions when compared to that from mammals, birds and shark. Some fish myoglobins contain a cysteine residue which could influence the susceptibility of myoglobin to oxidation (Brown, 1961). In general, fish myoglobins are more readily oxidized than mammalian myoglobins (Haard, 1992).

Whiteness is a critical factor determining the quality of surimi gels, and myoglobin and hemoglobin can influence this important parameter (Chen, 2002). In general, both heme proteins are removed during the washing process of fish mince, leading to increased whiteness. However, heme proteins become less soluble as fish quality deteriorates with time *post-mortem* (Chen, 2003). Prolonged iced storage decreased myoglobin extraction efficiency in sardine and mackerel muscle due to apparent insolubility of myoglobin caused by oxidation (Chaijan *et al*., 2005a). Chen (2003) also reported that frozen storage decreased the solubility of milkfish myoglobin, resulting in a greater myoglobin content retained in washed milkfish muscle. After capture, fish are normally kept in ice prior to unloading. During this stage, discoloration of muscle can occur and binding of pigments to muscle proteins also takes place. Denaturation and/or oxidation of myoglobin may induce the binding of myoglobin with structural muscle proteins (Chen, 2002; Ochiai *et al*., 2001). Furthermore, metmyoglobin has been reported to cross-link with myosin in the presence of hydrogen peroxide, a potential by-product of oxymyoglobin oxidation (Hanan and Shaklai, 1995). As a consequence, surimi produced from aged fish, especially dark-fleshed species, would be expected to be discolored. Conversion of oxymyoglobin to metmyoglobin is associated with beef discoloration (O'Grady *et al*., 2001; Faustman *et al*., 1992). This phenomenon is governed by a variety of factors including pH, temperature, ionic strength and oxygen consumption (Renerre and Labas, 1978). Metmyoglobin formation is positively correlated with lipid oxidation (Lee *et al*., 2003a; Chan *et al*., 1997). During improper handling, especially at abusive temperatures, or processing in the presence of high salt concentrations, the interaction of myoglobin and other muscle proteins can occur and affect color. The objective of this study was to investigate the role of ionic strength and temperature on the interaction between yellowfin tuna myoglobin and myofibrillar proteins from bluefish, which has been used for surimi production even to a small portion (Ashie and Simpson, 1996), in a model system.

7.3 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. Whole bluefish were immediately washed, filleted and skinned. Muscles were collected and used for natural actomyosin (NAM) preparation. The longissimus muscles of yellowfin tuna were cut into 0.2 kg pieces and stored at -20 $^\circ$C until required for myoglobin isolation.

Extraction and purification of myoglobin from tuna muscle

Extraction and purification of myoglobin were performed according to Trout and Gutzke (1996) with slight modification. The yellowfin tuna longissimus muscle (100 g) was coarsely minced 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 $\times g$ for 10 min at 4°C using an 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 obtain 65% saturation and the suspension was stirred for 60 min at 4°C. The suspension was centrifuged at 18,000 $\times g$ 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 20,000 $\times g$ 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, which was referred to as starting buffer. The mixture was then dialyzed against 10 vol of starting buffer for 24 h at 4°C. The dialysate was immediately applied to a Sephacryl S-200HR column (2.5 × 100 cm) previously equilibrated with the starting buffer. The separation was conducted at a flow rate of 60 ml/h and 5-ml fractions were collected.

During purification, the fractions 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 the myoglobin substrate.

Preparation of oxymyoglobin

Oxymyoglobin was prepared by hydrosulfite-mediated reaction of purified tuna myoglobin (Brown and Mebine, 1969), and residual hydrosulfite was removed by dialysis the sample against 10 volumes of cold 10 mM phosphate buffer, pH 6.5 for 24 h. 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 of chilled 0.6 M KCl, pH 7.0 (4°C) for 4 min. The homogenate was maintained in an ice bath and then centrifuged at 5,000 $\times g$ 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 $5,000 \times g$ for 20 min at 4°C, and the pellet 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 $5,000 \times g$ 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.

Effect of ionic strength on the interaction between fish myoglobin and NAM

Tuna oxymyoglobin (0.5 mg/ml) dissolved in 10 mM phosphate buffer, pH 6.5, containing KCl at concentrations of 0, 0.3 or 0.6 M was mixed with bluefish NAM (5 mg/ml) dispersed in the same buffer at a ratio of 1:1 (v/v). The oxidation of oxymyoglobin was measured after 3 h at 4 $^{\circ}$C. After washing with 3 vol cold 10 mM phosphate buffer, pH 6.5, and centrifuging at $5,000 \times g$ for 30 min at 4°C to remove the unbound myoglobin using the RC-5B centrifuge (Sorvall, Norwalk, CT, USA), the pellet was analyzed for whiteness, Ca^{2+}-ATPase activity, and protein patterns on SDS-PAGE. The myoglobin content in the supernatant was determined as mentioned below and the percentage of bound myoglobin was calculated via difference.

Effect of temperature on the interaction between fish myoglobin and NAM

Tuna oxymyoglobin (0.5 mg/ml) was dissolved in 10 mM phosphate buffer, pH 6.5, containing 0.6 M KCl, and mixed with bluefish NAM (5 mg/ml) solubilized in the same buffer at a ratio of 1:1 (v/v). The oxidation of oxymyoglobin was determined after incubation at 4 or 25°C for 3 h. To remove the unbound myoglobin, 3 vol of cold 10 mM phosphate buffer, pH 6.5, was added to the solution, mixed, and centrifuged at $5,000 \times g$ for 30 min at 4°C. Whiteness, Ca^{2+}-ATPase activity and protein patterns of the pellet were determined as described below. Myoglobin concentration was measured in the supernatant, and the percentage of bound myoglobin was then calculated via difference.

Determination of oxymyoglobin oxidation

The oxidation of oxymyoglobin during incubation with and without NAM was determined spectrophotometrically by scanning from 650 to 450 nm with a diffuse-integrating sphere attached to a Shimadzu UV-2100 spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD, USA). 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 NAM-myoglobin 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 consisting of myosin (206 kDa), $\square$-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 for reference purposes.

Determination of Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activity of NAM and NAM-myoglobin mixture was determined according to Benjakul *et al.* (1997). NAM in both 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 combined with 0.6 ml of 0.5 M Tris-maleate, pH 7.0, 1 ml of 0.1 M $CaCl_2$, and deionized water 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 at 25°C and terminated at 8.0 min by adding 5 ml of chilled 15% (w/v) trichloroacetic acid. The reaction mixture was centrifuged at 3,500 $\times g$ 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 pellet 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) as follows:

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

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). The T-test was used to evaluate the effect of temperature on the interaction between fish myoglobin and myofibrillar proteins. Statistical analysis was performed using the Statistical Package for Social Science (SPSS 10.0 for windows, SPSS Inc., Chicago, IL).

7.4 Results and Discussion

Effect of ionic strength on the interaction between fish myoglobin and NAM

The proportion of myoglobin bound to NAM was greater at 0.6 M KCl than 0 or 0.3 M KCl ($P<0.05$; Figure 29). With 0 and 0.3 M KCl, 19.74% and 20.72% of myoglobin was bound to NAM, respectively. The increase in bound myoglobin content at greater ionic strength was probably associated with increased solubility of NAM and the potential for interaction with

myoglobin (Thawornchinsombut and Park, 2005). Previously, several investigators reported that as salt concentration is increased, myosin molecules are released from thick filaments of the myofibrils, dispersed in the solution as monomers and partially unfolded (Lin and Park, 1998; Ishioroshi *et al.*, 1983). Copeland (1994) noted that when a protein unfolds, amino acid residues originally buried in the nonpolar interior of the protein become exposed to the polar aqueous solvent. Increasing KCl concentration of the aqueous myoglobin-NAM system would be expected to influence the conformation of both NAM and myoglobin and potentially favor the aggregation of myoglobin with myofibrillar proteins via hydrophobic interactions. In the absence of KCl, NAM was only slightly soluble (Lin and Park, 1998), but myoglobin would be completely solubilized; the opportunity for binding with NAM would be expected to be considerably less than at 0.6 M KCl.
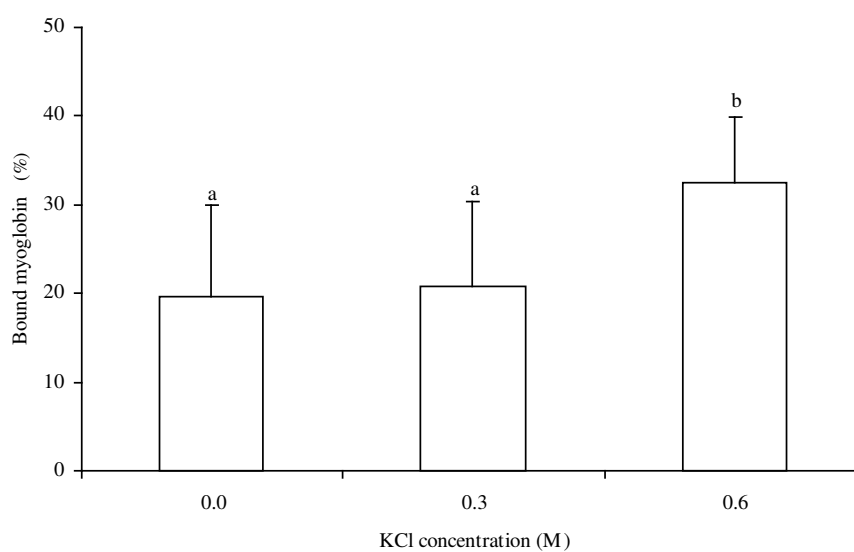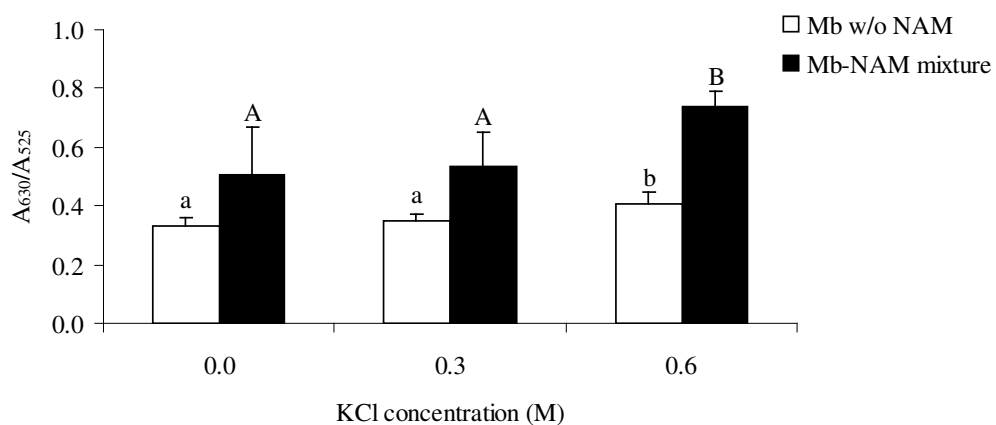

Figure 29. The effect of KCl concentration on bound myoglobin content (%) in a fish myoglobin-NAM model system. Bars indicate standard deviation from ten determinations. Different letters indicate significant differences ($P<0.05$).

Oxymyoglobin oxidation in assays with and without NAM at different KCl concentrations (0, 0.3 and 0.6 M KCl) is presented in Figure 30. The formation of metmyoglobin

(A_{630}/A_{525}) in both myoglobin controls (without NAM) and myoglobin-NAM mixtures increased at high ionic strength (0.6 M KCl) ($P<0.05$). No differences in metmyoglobin formation were observed in myoglobin controls and myoglobin-NAM mixtures at 0 and 0.3 M KCl ($P>0.05$). A greater A_{630}/A_{525} ratio was observed with myoglobin in the presence of NAM and 0.6 M KCl. The basis for this could be related to salt concentration effects on myoglobin oxidation (Trout, 1990), and/or interaction between myoglobin and NAM resulting in conformational changes that would predispose myoglobin to oxidation (Eder, 1996).
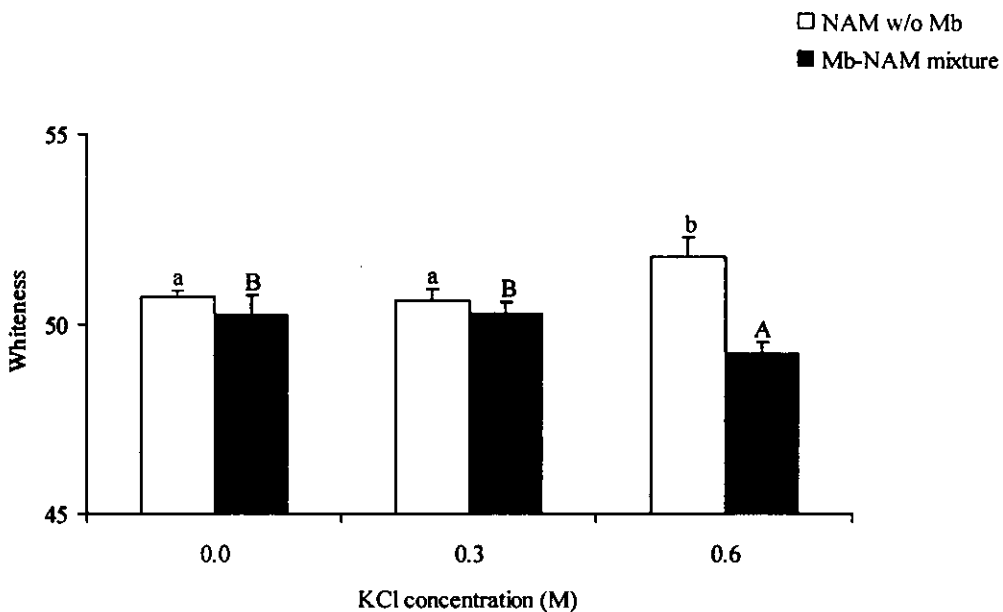

Figure 30. The effect of KCl concentrations on metmyoglobin formation (A_{630}/A_{525}) in myoglobin (□) and myoglobin-NAM mixture (□). Bars indicate standard deviation from ten determinations. Different letters, or different letter case among the KCl concentrations indicate significant differences ($P<0.05$).
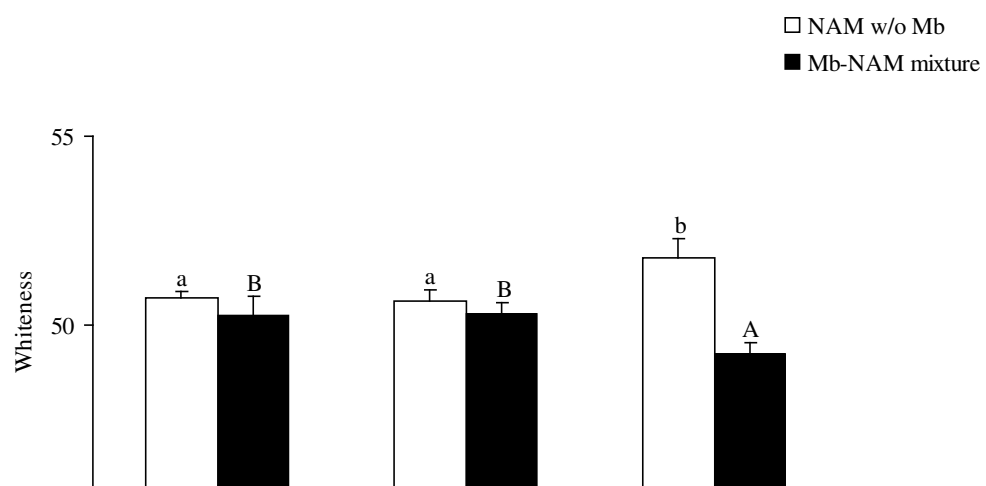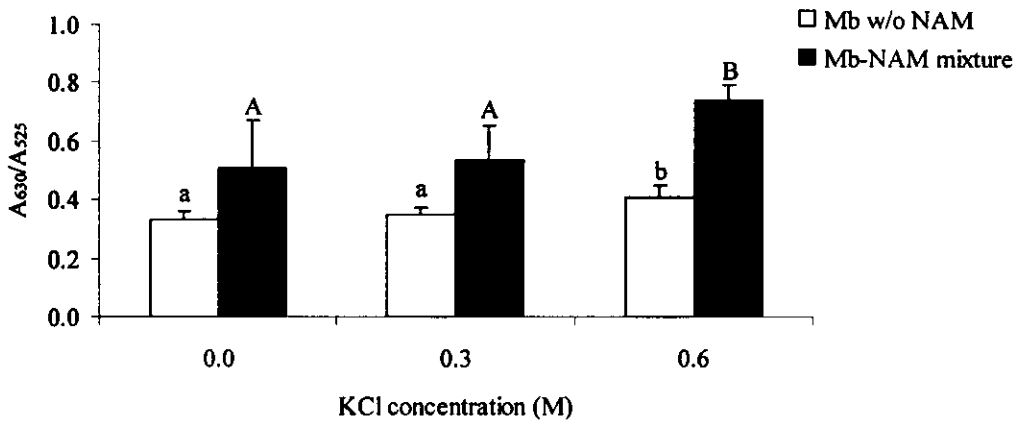
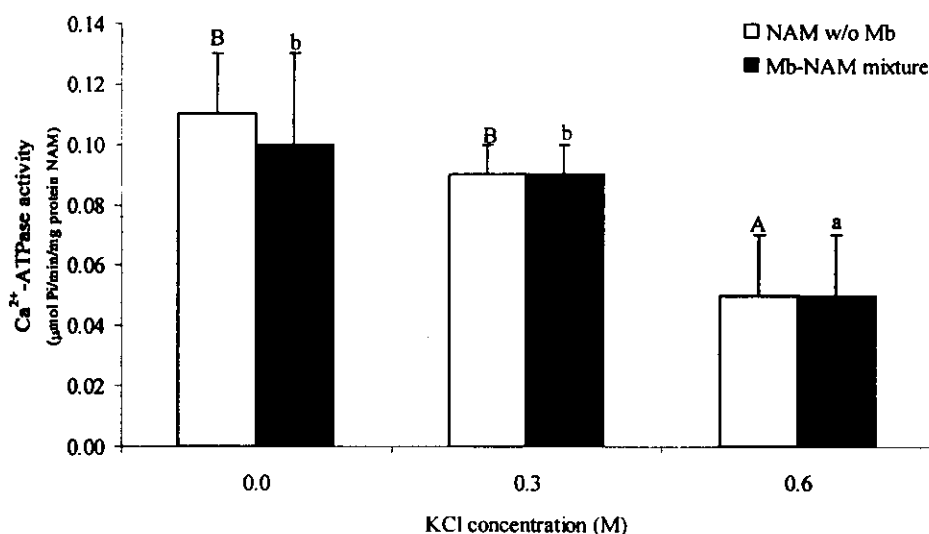**Figure 30.** The effect of KCl concentrations on metmyoglobin formation (A_{630}/A_{525}) in myoglobin ($\square$) and myoglobin–NAM mixture ($\blacksquare$). Bars indicate standard deviation from ten determinations. Different letters, or different letter case among the KCl concentrations indicate significant differences ($P<0.05$).

Figure 31. The effect of KCl concentrations on whiteness of NAM alone ($\square$) and myoglobin–NAM mixture ($\blacksquare$). Bars indicate standard deviation from ten determinations. Different letters, or different letter case among the KCl concentrations indicate significant differences ($P<0.05$).

The whiteness of NAM with and without myoglobin addition is presented in Figure 31. Whiteness of NAM alone increased gradually in the presence of 0.6 M KCl ($P<0.05$) consistent with the known solubility behavior of NAM. Lin and Park (1998) reported that the solubility of salmon myosin increased with increasing KCl concentrations up to 0.5 M and remained constant from 0.5 M to 1.0 M. The increased solubility of NAM at high KCl concentration might have arisen from the increased protein-solvent interactions. Consequently, the solubilized NAM was translucent and associated with greater L* values as well as whiteness. When NAM was incubated with myoglobin and subsequently analyzed, whiteness decreased. This result suggested that some myoglobin did bind and remained bound following washing. Myoglobin redox state could also influence NAM discoloration. The lowest whiteness values were recorded at 0.6 M KCl at which greater metmyoglobin formation (Figure 30) and bound myoglobin (Figure 29) were also observed.
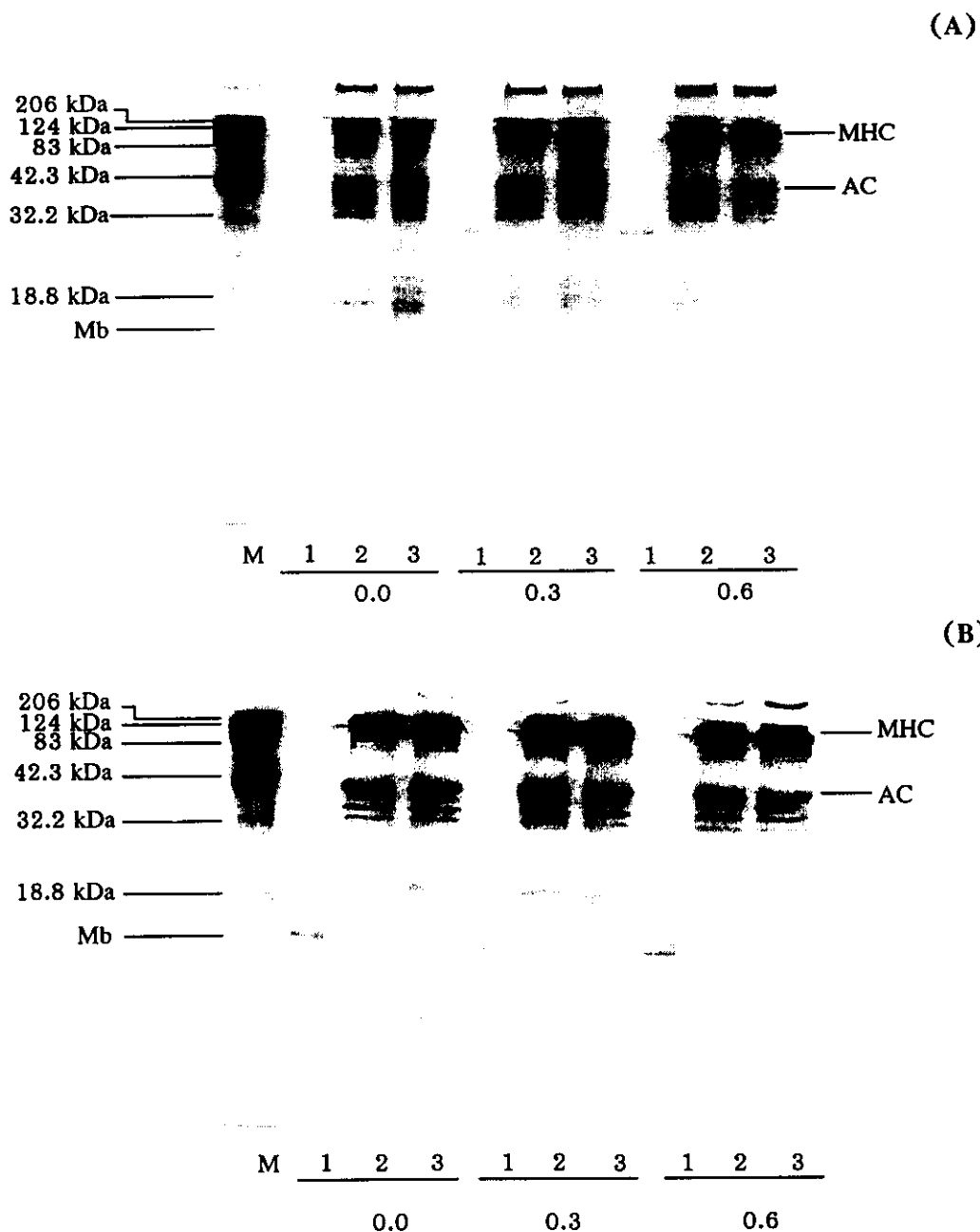

Figure 32. The effect of KCl concentrations on Ca^{2+}-ATPase activity of NAM ($\square$) and myoglobin-NAM mixture ($\blacksquare$). Bars indicate standard deviation from ten determinations. Different letters, or different letter case among the KCl concentrations indicate significant differences ($P<0.05$).

Ca^{2+}-ATPase activity decreased in both NAM controls and NAM-myoglobin at 0.6 M KCl ($P<0.05$; Figure 32). The greatest Ca^{2+}-ATPase activity was observed in NAM controls without KCl ($P<0.05$). Myofibrillar ATPase activities have

been widely used as a measure of actomyosin integrity (Roura *et al.*, 1990). Our results suggested that the loss of myosin Ca^{2+}-ATPase activity occurred and could have resulted from loss of protein integrity induced by high ionic strength. KCl would modify the charge of proteins, leading to molecular repulsion and subsequent conformational changes. At a given KCl concentration, the Ca^{2+}-ATPase activity of NAM controls and NAM-myoglobin mixtures was not markedly different ($P>0.05$). This result suggested that any myoglobin that did bind to myosin likely did so at domains other than myosin heads which possess the ATPase activity.

The SDS-PAGE patterns of myoglobin, NAM, and myoglobin-NAM mixtures under non-reducing and reducing conditions are presented in Figures 33A and 33B, respectively. In the presence of 0.6 M KCl, reduction in intensity of myosin heavy chaing (MHC) and actin bands was observed with the concomitant fading of the myoglobin band (Figure 33A, 33B). From this result, it was suggested that myoglobin-NAM interaction occurred via non-disulfide bonds. Both MHC and actin could bind with myoglobin, leading to the formation of a larger molecular weight aggregate; aggregates were observed with increased concentrations of KCl.

(A)

206 kDa
124 kDa
83 kDa
42.3 kDa
32.2 kDa

——MHC

—— AC

18.8 kDa
Mb

M   1   2   3   1   2   3   1   2   3
        0.0   0.3   0.6

(B)

206 kDa
124 kDa
83 kDa
42.3 kDa
32.2 kDa

—— MHC

—— AC

18.8 kDa
Mb

M   1   2   3   1   2   3   1   2   3
       0.0   0.3   0.6

Figure 33. SDS–PAGE patterns of myoglobin, NAM alone, and myoglobin–NAM mixture at different KCl concentrations under non-reducing (A), and reducing conditions (B). M, molecular weight standards; 1, myoglobin; 2, NAM; 3, 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 temperature on the interaction between fish myoglobin and NAM**

The interaction between myoglobin and NAM in 10 mM phosphate, pH 6.5, and containing 0.6 KCl was investigated at 4 and 25°C (Figure 34). The quantity of bound myoglobin at 25°C was approximately 2.5 times greater than that at 4°C ($P<0.05$). Accordingly, the rate of this interaction was temperature-dependent. It has been reported that higher temperatures can cause partial or even pronounced denaturation of proteins (Boye *et al.*, 1997). At 25°C, it is possible that NAM experienced conformational changes sufficient to increase its interactions with myoglobin. In addition, the higher temperature would have favored greater molecular mobility and the likelihood for NAM:myoglobin interaction. Both of these possibilities would have contributed to greater bound myoglobin at 25°C than at 4°C (Figure 34).
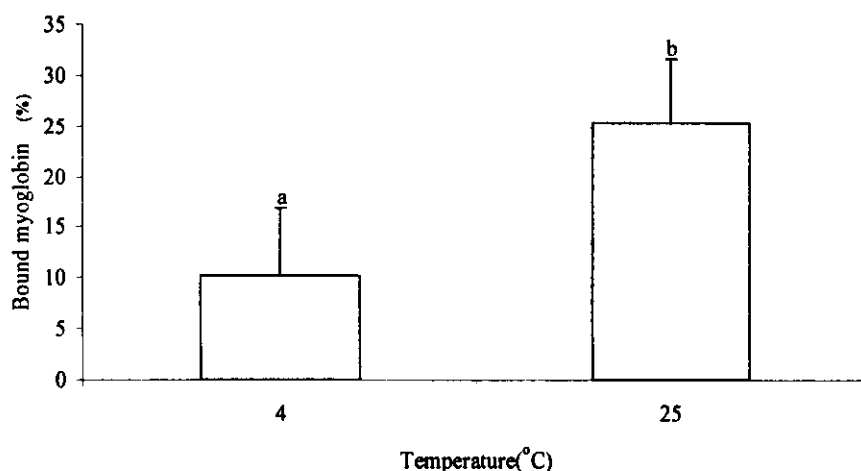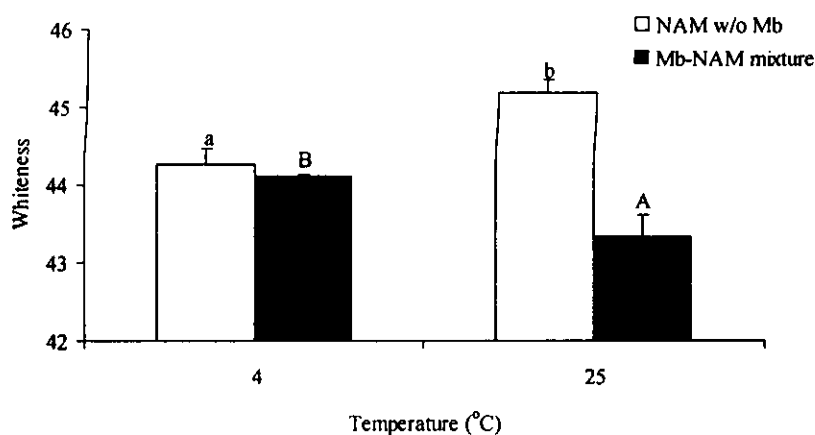

Figure 34. The effect of temperature on bound myoglobin content (%) in a fish myoglobin–NAM model system. Bars indicate standard deviation from ten determinations. Different letters indicate significant differences ($P<0.05$).

Metmyoglobin formation in NAM-myoglobin mixtures was greater at 25°C than 4°C ($P<0.05$; Figure 35). It is well known that myoglobin oxidation increases rapidly with increased temperature. Brown and Mebine (1969) reported that the autoxidation of tuna myoglobin was very temperature sensitive and reported a Q_{10} value of approximately 5 between -2°C and 22°C. The A_{630}/A_{525} ratio was greater for myoglobin mixed with NAM than myoglobin alone (Figure 35). The interaction between myoglobin

and NAM could induce slight changes in conformation that would predispose the protein to heme oxidation.
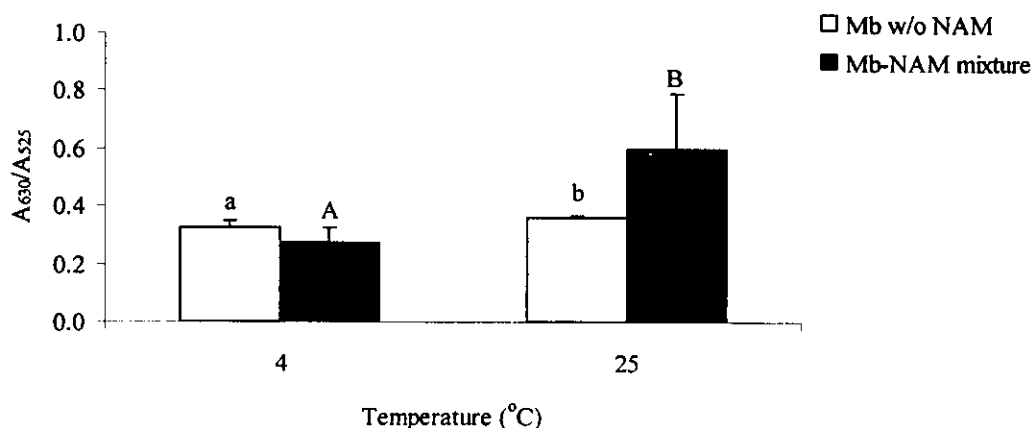
Figure 35. The effect of temperature on metmyoglobin formation (A_{630}/A_{525}) in myoglobin $(\square)$ and myoglobin-NAM mixture $(\blacksquare)$. Bars indicate standard deviation from ten determinations. Different letters, or different letter case among temperatures indicate significant differences $(P<0.05)$.

Figure 36. The effect of temperature on whiteness of NAM alone $(\square)$ and myoglobin-NAM mixture $(\blacksquare)$. Bars indicate standard deviation from ten determinations. Different letters, or different letter case among temperatures indicate significant differences $(P<0.05)$.

NAM whiteness was generally greater at $25^{\circ}C$ than that at $4^{\circ}C$ (Figure 36). At $25^{\circ}C$, a slightly greater solubility of NAM might occur and result in a more

translucent appearance leading to the greater associated L* value. However, at the higher temperature the NAM–myoglobin mixture demonstrated decreased whiteness compared with NAM alone ($P<0.05$; Figure 36). The binding of myoglobin to NAM and/or the formation of metmyoglobin after the interaction between oxymyoglobin and NAM contributed to decreased whiteness. The decreased whiteness observed was consistent with greater bound myoglobin (Figure 34), and A_{630}/A_{525} ratios (Figure 35) of myoglobin–NAM mixtures incubated at 25°C versus 4°C.
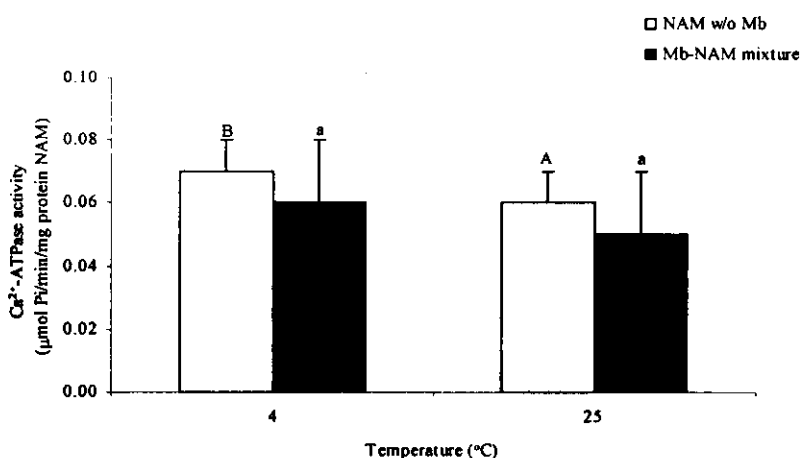

Figure 37. The effect of temperature on Ca^{2+}-ATPase activity of NAM alone (□) and myoglobin–NAM mixture (■). Bars indicate standard deviation from ten determinations. Different letters, or different letter case among temperatures indicate significant differences ($P<0.05$).

Ca^{2+}-ATPase activity of NAM was less at 25°C than at 4°C ($P<0.05$) (Figure 37), suggesting greater loss of myosin integrity at the higher temperature. In the presence of myoglobin, Ca^{2+}-ATPase activity tended to be lowered relative to controls without myoglobin. No differences in Ca^{2+}-ATPase activity were found in myoglobin–NAM mixtures incubated at the two temperatures ($P>0.05$). The formation of high-molecular–weight aggregates was observed in SDS-PAGE (Figures 38A and 38B) and appeared to be more significant at 25°C than at 4°C. If myoglobin was binding to myosin, then it did so at domains other than the head portion because Ca^{2+}-ATPase activity was not affected by the presence of myoglobin (Figure 37).
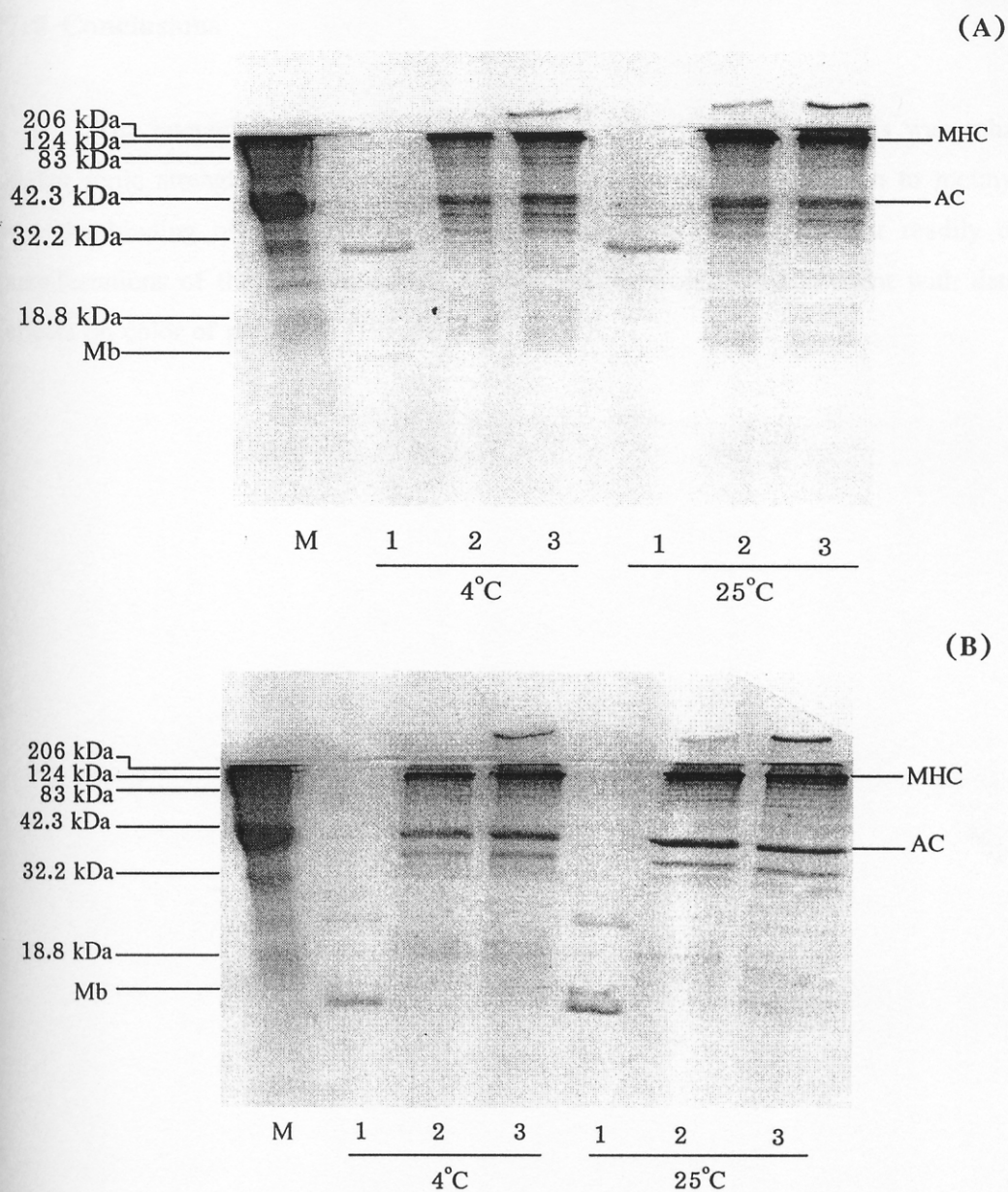
Figure 38. SDS-PAGE patterns of myoglobin, NAM, and myoglobin-NAM mixture at different temperatures under non-reducing (A) and reducing conditions (B). M, molecular weight standards; 1, myoglobin; 2, NAM; 3, myoglobin-NAM mixture. MHC, myosin heavy chain; AC, actin. Myoglobin protein loaded onto SDS-PAGE 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.

7.5 Conclusions

The interaction between fish myoglobin and myofibrillar proteins was enhanced at higher ionic strength and temperature. The oxidation of oxymyoglobin to metmyoglobin and the binding of myoglobin to myofibrillar proteins were the most readily observed manifestations of these interactions. These changes would be consistent with detrimental effects on color of processed fish muscle.