CHAPTER 8

EFFECT OF INCUBATION TIME ON INTERACTION BETWEEN FISH MYOGLOBIN AND MYOFIBRILLAR PROTEINS

8.1 Abstract

Interaction between fish myoglobin and myofibrillar proteins in a model system was investigated at 4°C. Increases in metmyoglobin formation and the relative content of bound myoglobin in a myoglobin-natural actomyosin model were observed as were decreases in whiteness and Ca^{2+}-ATPase activity with incubation time (*P*<0.05). During the first 6 h, myoglobin bound preferably to myosin at domains other than the head, or other myofibrils in natural actomyosin (NAM), as evidenced by the remaining ATPase activity. The potential binding of myoglobin to myosin heads appeared to occur after 24 h incubation as evidenced by the marked decrease in Ca^{2+}-ATPase activity of the NAM-myoglobin mixture when compared to that of NAM alone (*P*<0.05). Interaction between fish myoglobin and myofibrillar proteins was more pronounced when the storage time increased as indicated by the increasing formation of high-molecular-weight aggregate (>206 kDa). Electrophoretic study revealed that disulfide bonds did not involve in myoglobin-NAM interactions.

8.2 Introduction

Myoglobin, a monomeric globular heme protein, is responsible for the pigmentation of meat (Faustman and Phillips, 2001). Its concentration in muscle tissue is related to a variety of factors including fiber type, muscular activity, oxygen availability, blood circulation and the age of animal (Haard, 1992). For surimi processing, myoglobin and hemoglobin can strongly affect whiteness, one of the principal factors determining the quality of surimi gels (Chen, 2002). In general, hemoglobin contributes less to the appearance of postmortem fish muscle because it is lost during handling and storage, whereas myoglobin is

retained by the intracellular structure (Haard *et al*., 1994). During surimi processing, both heme proteins can be removed from fish muscle mince during the washing process, leading to increased whiteness. However, heme proteins become less soluble as the fish muscle deteriorations and this appears to affect the efficiency of heme protein removal (Chen, 2003). Denaturation of myoglobin, before or during processing, can also result in its binding to myofibrillar proteins, reduce the efficiency of the washing process, and discolor the subsequent surimi product (Lanier, 2000). It is difficult to leach all of the myoglobin from dark fish muscle because it resides within the muscle cells (Lanier, 2000). Recently, Chaijan *et al*. (2005a) reported that prolonged iced storage decreased myoglobin extracting efficiency in sardine and mackerel muscle due to the insolubility of myoglobin caused by the oxidation during storage. In a related observation, frozen storage resulted in decreased solubility of milkfish myoglobin, resulting in more myoglobin being retained in washed milkfish muscle (Chen, 2003). Conversion of oxymyoglobin to metmyoglobin is associated with discoloration of muscle foods (O'Grady *et al*., 2001; Faustman *et al*., 1992). After capture, fish are normally kept in ice prior to off-loading. During this stage, discoloration of muscle can occur and binding of pigments to muscle also takes place. Denaturation and/or oxidation of myoglobin may enhance its ability to bind muscle proteins (Chen, 2002; Ochiai *et al*., 2001). Metmyoglobin was shown 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 fish species, can be expected to be relatively discolored. Improper handling, especially when combined with extended storage may negatively affect the color of processed fish muscle presumably due to interaction of myoglobin with muscle proteins (Chen, 2003; Lanier, 2000). Therefore, the objective of this study was to investigate the interaction between fish myoglobin and myofibrillar proteins as a function of time in a model system at refrigerated temperature (4°C).

8.3 Materials and Methods

Chemicals

Sephacryl S-200HR, sodium dodecyl sulfate (SDS), β-mercaptoethanol (β-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 under vacuum packaging at -20°C until used 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$\times g$ 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$\times g$ 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$\times g$ 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, 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 $\times$ 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 the myoglobin substrate.

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$\times g$ 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$\times g$ 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$\times g$ 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.

Study on the interaction between fish myoglobin and NAM during the incubation at 4°C

Tuna oxymyoglobin (0.5 mg/ml) prepared 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 a ratio of 1:1 (v/v). Oxymyoglobin oxidation was determined after incubation at 4°C for 0, 3, 6 and 24 h. 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 by SDS-PAGE. The myoglobin content in the supernatant was determined, and the percentage of bound Mb was 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 washed NAM-myoglobin mixture was determined according to Benjakul *et al*. (1997). NAM and NAM-myoglobin mixture was diluted to 2.5 to 8 mg/ml with 0.6 M KCl, pH 7.0. Diluted samples (1 ml) were mixed with 0.6 ml of 0.5 M Tris-maleate, pH 7.0, and 1 ml of 0.1 M $CaCl_2$. Deionized water was added to obtain a total volume of 9.5 ml. To initiate the reaction, 0.5 ml of 20 mM adenosine 5'-triphosphate (ATP) solution was added. The reaction proceeded for 8 min at 25°C and was 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, IL).

8.4 Results and Discussion

Purification of myoglobin from tuna muscle

A crude myoglobin extract was subjected to 65-100% ammonium sulfate precipitation. The pellet obtained was then further purified by gel filtration chromatography using the Sephacryl S-200HR column. A major peak with A_{540} representing myoglobin was observed (Figure 39).

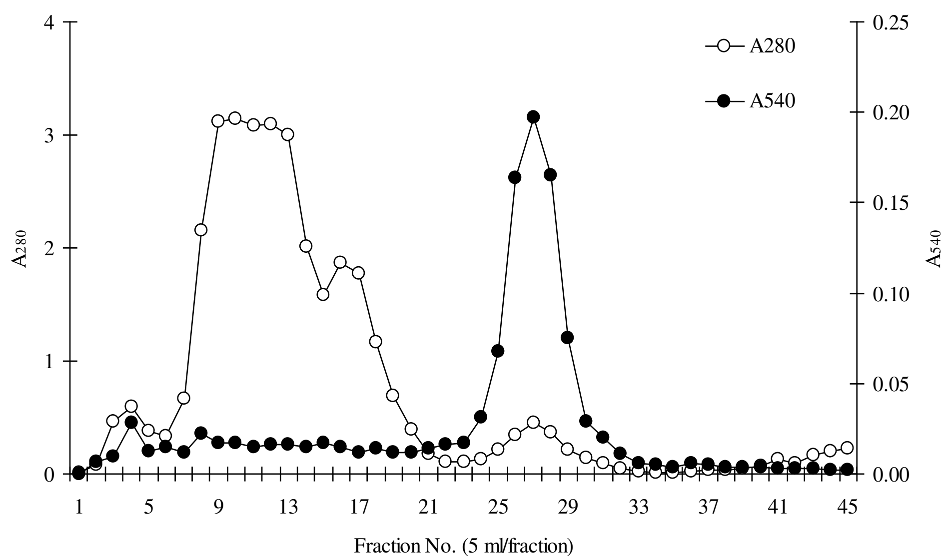


Figure 39. Elution profile of tuna myoglobin on the Sephacryl S-200HR. The column was equilibrated and eluted with 5 mM Tris-HCl buffer containing 1 mM EDTA, pH 8.0. The flow rate was 60 ml/h and 5-ml fractions were collected.

From the chromatogram, a small peak with A_{540} was also present (~fractions 3-4) and likely contained hemoglobin and other high-molecular-weight proteins. The SDS-PAGE patterns of tuna myoglobin extract during purification process are shown in Figure 40. Tuna longissimus muscle extract contained a large number of proteins. Proteins with molecular weights ranging from 35 to 45 kDa predominated (Figure 40, lane 2). After ammonium sulfate precipitation (65-100% saturation), the protein bands with molecular weights of 42.3, 40, 35 and 28 kDa became more intense (Figure 40, lane 3). After gel filtration, proteins with molecular weight higher than 14.1 kDa were completely removed and a major protein band with the molecular weight of 14.1 kDa appeared (lane 4). Densitometric analysis revealed that the myoglobin band represented approximately 97.5% of the total protein contained in the pooled Sephacryl S-200HR fraction. The molecular weight of myoglobin was estimated to be 14.1 kDa, which was lower than that of equine myoglobin (17.4 kDa) (Figure 40, lane 5).

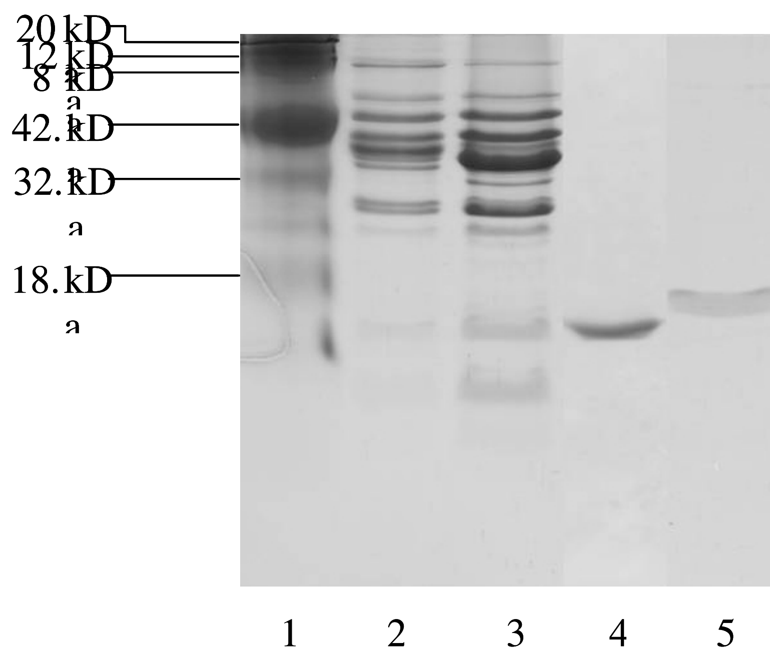


Figure 40. SDS-PAGE pattern of partially purified myoglobin from tuna longissimus muscle. Lane 1, molecular-weight standard; lane 2, crude extract; lane 3, ammonium sulfate precipitated fraction; lane 4, Sephacryl S-200HR fraction; lane 5, equine myoglobin.

The mass of yellowfin tuna myoglobin approximated molecular weight of bigeye tuna, milkfish, mackerel and sardine, which were reported to be 15,540 daltons (Ueki and Ochiai, 2004), 15,900 daltons (Chen and Chow, 2001), 14,900 daltons (Yamaguchi *et al*., 1979) and 15,300 daltons (Chaijan *et al*., 2005b), respectively. Satterlee and Zarchariah (1972) reported that the molecular weights of myoglobin from porcine, ovine and sperm whale were 17,700, 17,100 and 17,000 daltons, respectively. While Renerre *et al*. (1992) found that bovine myoglobin was approximately 17,000 daltons. Generally, the molecular weights of fish myoglobins are less than those of mammalian myoglobins.

Effect of incubation time on bound myoglobin content of fish myoglobin-NAM model systems

The quantity of myoglobin bound to NAM increased with increasing incubation time (P<0.05; Figure 41), specifically by 59.6 and 71.6% when the mixture was incubated at 4°C for 3 and 6 h, respectively. After 24 h incubation, bound myoglobin increased approximately 3-fold, compared with that observed at 0 h The interaction between fish myoglobin and myofibrillar proteins was time dependent and may have resulted from conformational changes that facilitated protein:protein interactions.

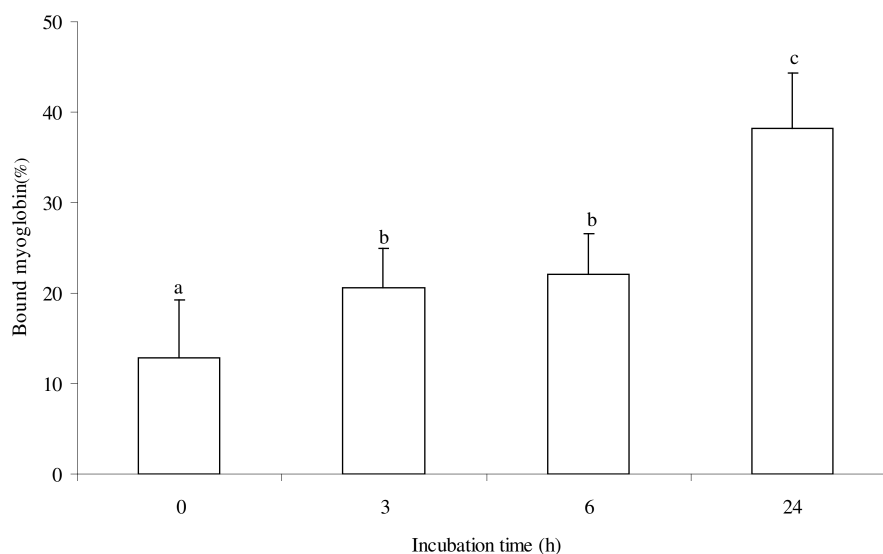


Figure 41. Bound myoglobin content (%) in fish myoglobin-NAM model system during incubation at 4°C. Bars represent the standard deviation from ten determinations. Different letters indicate significant differences (P<0.05).

However, myoglobin also bound to NAM even 0 h of incubation. Myofibrillar proteins and myoglobin completely solubilized in the presence of 0.6 M KCl and this was the prerequisite process for binding of myoglobin and myofibrillar proteins. Myoglobin bound to structural proteins would make it more difficult to remove myoglobin from muscle via washing. Chen (2003) reported that myoglobin extracting efficiency in milkfish muscle decreased with increased iced storage time.

Effect of incubation time on metmyoglobin formation of fish myoglobin and myoglobin-NAM model system

Oxymyoglobin oxidation in the presence or absence of NAM is presented in Figure 42. The formation of metmyoglobin (A_{630}/A_{525}) in both myoglobin (without NAM) and myoglobin-NAM mixtures was negligible during 6 h incubation (P>0.05). After 24 h incubation, metmyoglobin formation was increased 13.3%, over that present at 0 h.

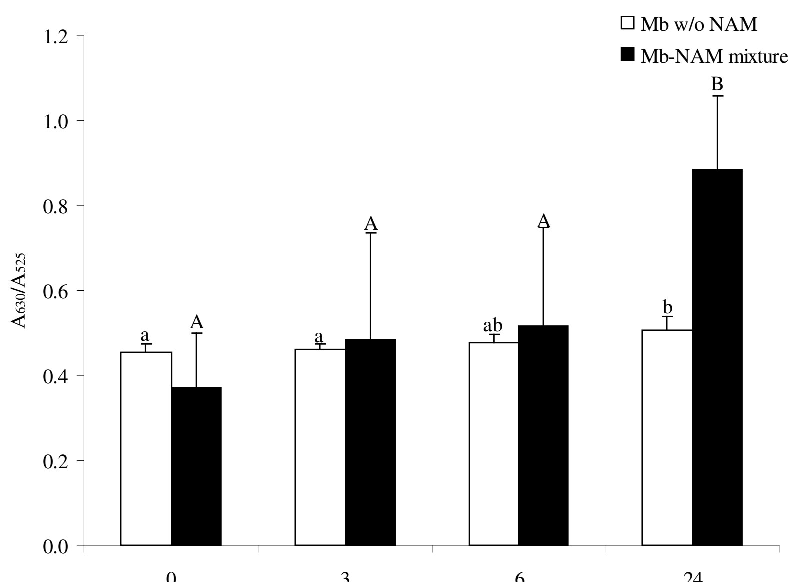
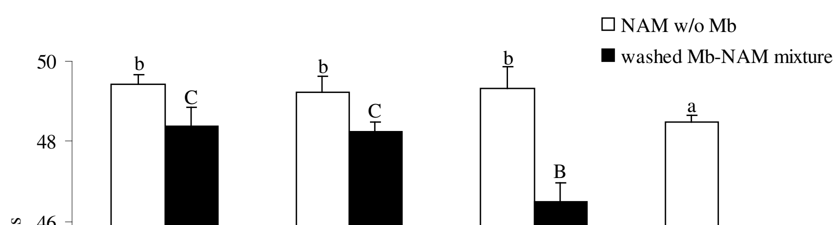


Figure 42. Metmyoglobin formation (A_{630}/A_{525}) in myoglobin (□) and myoglobin-NAM mixture (□) during incubation at 4°C. Bars represent the standard deviation from ten determinations. Different letters, or different letter case among incubation times indicate significant differences (P<0.05).

Many factors affect oxymyoglobin oxidation, including pH, salt concentration, temperature, (Trout, 1990); and duration of storage of muscle prior to processing (Chaijan *et al.*, 2005a; Chen, 2003). A greater A_{630}/A_{525} ratio indicating more metmyoglobin was observed for myoglobin in the presence than absence of NAM, 2.4 fold especially after 24 h incubation. Processing steps associated with surimi preparation would favor metmyoglobin formation and/or favor interaction between myoglobin and NAM that could favor additional metmyoglobin formation. The globin portion of myoglobin serves a critical role in protecting heme against oxidation (Xiong, 1997). If its conformation was altered even minimally, particularly with extended storage, it would be expected to oxidize. Eder (1996) reported that myoglobin was susceptible to oxidation when it lost the native conformation.

Effect of incubation time on whiteness of NAM and washed NAM-myoglobin model system

Whiteness values for NAM and NAM-myoglobin mixtures after washing are illustrated in Figure 43. In the absence of myoglobin, no differences in NAM whiteness were observed during 6 h incubation (P>0.05).



Effect of incubation time on Ca^{2+}-ATPase activity in NAM and washed NAM-myoglobin model system

Ca^{2+}-ATPase activity in both NAM and washed NAM-myoglobin mixture decreased sharply within the first 3 h following washing (P<0.05; Figure 44). No differences in Ca^{2+}-ATPase activity between NAM and washed NAM-myoglobin mixture were observed during 6 h in incubation. A marked decrease in activity of washed NAM-myoglobin mixture was observed at 24 h when compared to washed NAM alone (P<0.05). Myofibrillar ATPase activities have been used extensively as a measure of actomyosin integrity (Roura *et al*., 1990). Our result suggests that the denaturation and/or aggregation of myosin occurred progressively with increasing incubation. The lack of myoglobin effect during the first 6 h suggests that it bound preferably to domains of myosin or myofibrils other than the myosin heads which possess the ATPase activity. However, binding of myoglobin to myosin heads may have occurred after 24 h as demonstrated by the marked decrease in Ca^{2+}-ATPase activity of washed NAM-myoglobin mixture, compared to that of NAM alone.

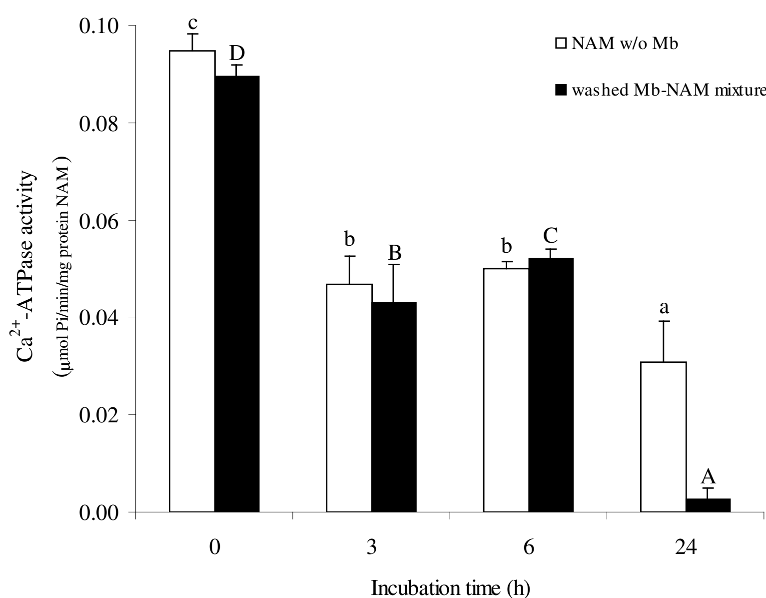


Figure 44. Ca^{2+}-ATPase activity of washed NAM (□) and washed myoglobin-NAM mixtures (□) during incubation at 4°C. Bars represent the standard deviation from ten determinations. Different letters or different letter case among incubation times indicate significant differences (P<0.05).

Effect of incubation time on SDS-PAGE patterns

The SDS-PAGE patterns of NAM and washed myoglobin-NAM mixtures under non-reducing and reducing conditions are presented in Figure 45A and 45B, respectively. At longer incubation times, a reduction in density of MHC and actin bands was observed with the concomitant disappearance of the myoglobin band (Figure 45A, B). Similar patterns were observed under non-reducing and reducing conditions suggesting that myoglobin-NAM protein interactions occurred via non-disulfide bonds.

(A)

(B)

Figure 45. SDS-PAGE patterns of myoglobin, washed NAM, and washed myoglobin-NAM mixture during incubation at 4°C 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.

8.5 Conclusions

The interaction between fish myoglobin and myofibrillar proteins was most pronounced at greater incubation times at 4°C. Oxymyoglobin oxidation to metmyoglobin occurred progressively, and the binding of myoglobin/metmyoglobin to myofibrillar proteins increased with incubation time. Thus, the extended *post-mortem* handling or storage of fish raw materials may result in increased binding between heme proteins and myofibrillar protein leading to greater discoloration (less white) of processed fish muscle.