CHAPTER 10

INTERACTION BETWEEN FISH MYOGLOBIN AND MYOSIN *IN VITRO*

10.1 Abstract

Interaction between tuna myoglobin and myosins from tuna and sardine was investigated in a model system at 4°C for up to 24 h. Both sardine and tuna myosins bound progressively with tuna myoglobin as the storage time increased (P<0.05). The soret absorption peak was noticeable in the myoglobin-myosin mixture. The oxidation of oxymyoglobin in the presence of myosin was generally greater than that was in the absence of myosin (P<0.05) Oxymyoglobin underwent oxidation to a higher extent in the presence of tuna myosin than sardine myosin (P<0.05). The interaction between fish myoglobin and myosin also caused the changes in reactive sulfhydryl content and altered the tryptophan fluorescent intensity. The loss in Ca^{2+}-ATPase activity of myosin varied with fish species and was governed by the myoglobin added. Thus, the interaction between fish myoglobin and myosin most likely occurred as a function of time and was species specific.

10.2 Introduction

Dark fleshed-fish species contained the high content of dark muscle comprising a considerable amount of lipids and sarcoplasmic proteins (Sikorski *et al*., 1990; Spinelli and Dassow, 1982). The presence of sarcoplasmic proteins and lipids of dark muscle is associated with its poorer gelation characteristics, compared with light muscle (Chen, 2002; Ochiai *et al*., 2001; Hultin and Kelleher 2000a; Haard *et al*., 1994). Sarcoplasmic proteins had an adverse effect on the strength, deformability (Haard *et al*., 1994) and color (Chaijan *et al*., 2004; Chen *et al*., 1997) of fish myofibril protein gels. Myoglobin is the predominant pigment protein in sarcoplasmic fraction of fish dark muscle (Hashimoto *et al*., 1979.) and contributes to the lowered

whiteness of surimi gel (Chen, 2002). Generally, it is difficult to wash all myoglobin from dark fleshed-fish muscle because it resides within the muscle cells (Lanier 2000; Haard *et al*., 1994). Normally, myoglobin 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 (Chaijan *et al*., 2005a; Chen, 2003). Denaturation of the myoglobin and/or myofibrillar proteins, before or during processing, can also cause their cross-linking, resulting in the discoloration of the surimi (Lanier, 2000). Recently, Chaijan *et al*. (2006) reported that the interaction between fish myoglobin and natural actomyosin was enhanced at higher ionic strength and temperature and the binding was augmented with increasing incubation times.

After capture, fish are normally kept in ice prior to unloading (Chaijan *et al*., 2005a; Emilia and Santos-Yap, 1995). During this stage, discoloration of muscle and binding of pigments to muscle generally occur (Lanier, 2000; Sikorski, 1994). However, no information regarding the interaction between myosin, which is the major myofibrillar protein in fish muscle, and myoglobin has been reported. Therefore, this study aimed to monitor the interaction between fish myoglobin and myosin in a model system at 4°C for different incubation times.

10.3 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

Sardine (*Sardinella gibbosa*) and tuna (*Thunnus albacares*) were used in this study. Sardine with an average weight of 55-60 g were caught from Songkhla-Pattani Coast along

the Gulf of Thailand. The fish, off-loaded approximately 12 h after capture, were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 2 h. Fish (1 kg) were immediately washed, filleted and skinned. Ordinary muscles were collected and used as the composite samples for myosin preparation. Yellowfin tuna with an average weight of 1.2 kg were obtained from Tropical Canning Co., Ltd. (Songkhla, Thailand.). The longissimus muscles of yellowfin tuna were cut into 0.2 kg pieces and stored at -20°C until required for myoglobin isolation and myosin preparation.

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 coarsely minced and mixed with 300 ml of cold extracting medium (10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and 25 g/l Triton X-100). The mixture was homogenized for 1 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). The homogenate was centrifuged at 5,000×*g* for 10 min at 4°C using an RC-5C plus 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×*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×*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, pH 8.0, containing 1 mM EDTA referred to as "starting buffer". The mixture was then dialyzed against 10 volumes of starting buffer for 24 h at 4°C. The dialysate was immediately applied to a Sephacryl S-100HR column (2.6 × 70 cm; Amersham Bioscience, Uppsala, Sweden) 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.

Preparation of oxymyoglobin

Oxymyoglobin was prepared by hydrosulfite-mediated reaction of purified tuna myoglobin (Brown and Mebine, 1969) and the 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).

Preparation of myosin

Myosin was extracted according to the method of Martone *et al*. (1986) as modified by Vissessanguan *et al*. (2000). All steps were performed at 4°C to minimize proteolysis and protein denaturation. Fish fillets were finely chopped and treated with 10 volumes of buffer A (0.10 M KCl, 1 mM PMSF, 10 μM E-64, 0.02% NaN_3 and 20 mM Tris-HCl, pH 7.5). After incubation on ice for 10 min with occasional stirring, the washed muscle was recovered by centrifugation at 1,000×*g* for 10 min. The pellet was suspended in 5 volumes of buffer B (0.45 M KCl, 5 mM □ME, 0.2 M $Mg(CH_3COO)_2$, 1 mM EGTA and 20 mM Tris-maleate, pH 6.8), and adenosine 5'-triphosphate (ATP) was added to obtain a final concentration of 10 mM. The mixture was kept on ice for 1 h with occasional stirring and centrifuged at 10,000×*g* for 15 min. Supernatant was collected and treated slowly with 25 volumes of 1 mM $NaHCO_3$, followed by incubation for 15 min on ice. Precipitated myosin was collected by centrifugation at 12,000×*g*, resuspended gently with 5 volumes of buffer C (0.50 M KCl, 5 mM □ME and 20 mM Tris-HCl, pH 7.5), and treated with 3 volumes of 1 mM $NaHCO_3$. $MgCl_2$ was also added to obtain a final concentration of 10 mM. The mixture was kept overnight on ice prior to centrifugation at 22,000×*g* for 15 min. Myosin, recovered as a pellet, was used immediately or stored at -20°C in 50% glycerol.

Study on the interaction between fish myoglobin and myosin at 4°C

Tuna oxymyoglobin (0.5 mg/ml) dissolved in 0.05 M potassium phosphate buffer containing 0.5 M KCl, pH 6.5 was mixed with tuna or sardine myosin (5 mg/ml) solubilized in the same buffer at a ratio of 1:1 (v/v). After incubation at 4°C for 24 h, the mixture was subjected to washing with 3 volumes of cold 10 mM phosphate buffer, pH 6.5, and centrifuge at 5,000 *X g* for 30 min at 4°C to remove the unbound myoglobin using the RC-5B centrifuge (Sorvall, Norwalk, CT, USA), the pellet obtained was dissolved with 0.05 M potassium phosphate buffer, pH 6.5 containing 0.5 M KCl. The solution was analyzed for absorption spectra, reactive sulfhydryl (SH) content, Ca^{2+}-ATPase activity and tryptophan fluorescence intensity. The oxymyoglobin oxidation in the supernatant was measured and the myoglobin content was also determined. The percentage of bound myoglobin was calculated via the difference.

Determination of oxymyoglobin oxidation

The oxidation of oxymyoglobin during incubation in the presence or the absence of myosin was determined spectrophotometrically. 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.

Determination of Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activity of myosin and washed myosin-myoglobin mixture was determined as described by Benjakul *et al*. (1997). Myosin and myosin-myoglobin mixture were diluted to 2.5 to 8 mg/ml with 0.05 M potassium phosphate buffer, pH 6.5, containing 0.5 M KCl. Diluted sample (1 ml) was 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 ATP solution was added. The reaction was run 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 *Xg* 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 protein/min. A blank solution was prepared by adding the chilled trichloroacetic acid prior to addition of ATP.

Determination of reactive SH content

Reactive SH content was measured using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) according to the method of Ellman (1959) as modified by Sompongse *et al*. (1996). Sample (0.5 ml, 4 mg/ml) was added to 4.5 ml of 0.2 M Tris-HCl buffer, pH 6.8. A 5 ml-aliquot of the mixture was taken and 0.5 ml of 0.1% DTNB solution was added. The mixture was incubated at 40°C for 25 min. Absorbance was measured at 412 nm using a Sherwood spectrophotometer 259 (Sherwood Scientific Ltd., Cambridge, UK). A blank was prepared by replacing the sample with 0.05 M potassium phosphate buffer, pH 6.5 containing 0.5 M KCl. SH content was calculated from the absorbance using the molar extinction of 13,600 $M^{-1}cm^{-1}$ and was expressed as mol/10^5 g protein.

Measurement of tryptophan fluorescence

Tryptophan fluorescence of myosin and myosin-myoglobin mixture was measured with a Jasco FP-6500 spectrofluorometer (Jasco, Tokyo, Japan) at an excitation wavelength of 280 nm and an emission wavelength of 325 nm according to the method of Chanthai *et al*. (1996).

Absorption spectra

The absorption spectra of myosin and washed myosin-myoglobin mixture was determined using a ND-1000 Nanodrop spectrophotometer (Nanodrop Technologies, Inc.,

Wilmington, DE, USA) as described by Chaijan et al. (2005a). The spectra were recorded from 350 to 450 nm at the scanning rate of 1,000 nm/min using 0.05 M potassium phosphate buffer, pH 6.5 containing 0.5 M KCl as a blank.

Statistical analysis

Data was 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).

10.4 Results and Discussion

Effect of myoglobin-myosin interaction on bound myoglobin content

Bound myoglobin content in myosin-myoglobin model systems is depicted in Figure 56. Both sardine and tuna myosins bound to tuna myoglobin progressively during incubation at 4°C for 24 h (P<0.05).

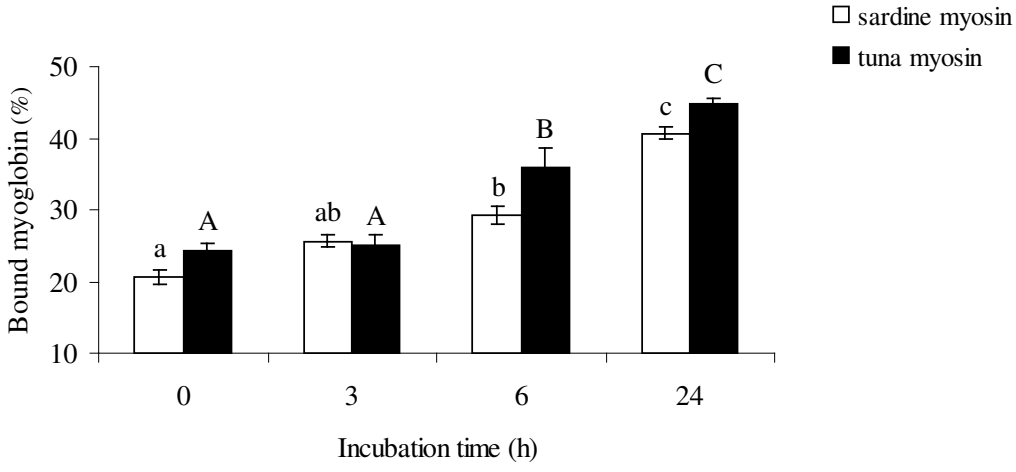


Figure 56. Bound myoglobin content (%) in fish myoglobin-myosin mixtures during incubation at 4°C. Bars represent the standard deviation from triplicate determinations. Different letters or different letter case within the same fish myosins indicate significant differences (P<0.05).

From the result, myoglobin could bind to myosin as both myoglobin and myosin were mixed instantaneously (0 h). At 0 h of incubation, the contents of tuna myoglobin bound to sardine myosin and to tuna myosin were 20.6 and 24.4%, respectively. The result suggested that both proteins could interact each other via some bondings regardless of protein conformational changes. The highest bound myoglobin content was observed in the myosin-myoglobin mixture after incubation for 24 h. With increasing incubation time at 4°C, the denaturation or unfolding of both myosin and myoglobin might occur in the way, which enhanced the interaction between those proteins. Generally, tuna myoglobin was adducted with tuna myosin to a greater extent, compared with sardine myosin. From the result, the interaction between fish myoglobin and myosin was most likely species specific and increased with increasing incubation time.

Effect of myoglobin-myosin interaction on metmyoglobin formation

The oxidation of oxymyoglobin increased with increasing incubation time in both the control myoglobin and myosin-myoglobin mixture (P<0.05) (Figure 57). The formation of metmyoglobin in the presence of myosin was generally higher than that without myosin. In the presence of tuna myosin, myoglobin underwent the oxidation more intensively, compared with the system containing sardine myosin.

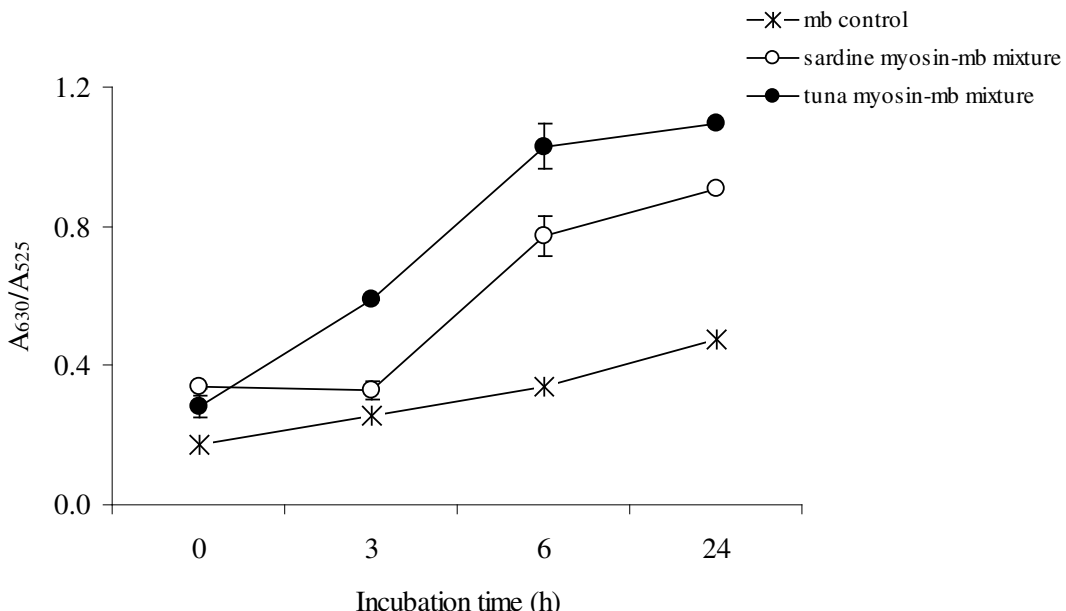


Figure 57. Metmyoglobin formation (A_{630}/A_{525}) in the myoglobin and myoglobin-myosin mixtures during incubation at 4°C. Bars represent the standard deviation from triplicate determinations.

After 24 h of incubation, the metmyoglobin formation in tuna myosin-tuna myoglobin mixture was 2.3-fold greater than that found in the control myoglobin, whereas the quantity of metmyoglobin in sardine myosin-tuna myoglobin mixture was approximately 1.9-fold higher than that observed in the control myoglobin. The result suggested that the conformation changes of globin could occur easily when myoglobin was adducted with myosin, especially from the same fish species. As a result, it might favor the oxidation of the heme molecule. Eder (1996) reported that myoglobin was susceptible to oxidation when it lost the native conformation.

Effect of myoglobin-myosin interaction on tryptophan fluorescence intensity

Tryptophan fluorescence intensity of myosin and myosin-myoglobin mixture during incubation at 4°C for 24 h is shown in Figure 58. Copeland (1994) pointed out that when a protein unfolds, amino acid residues that were buried in the nonpolar interior of the protein become exposed to the polar aqueous solvent.

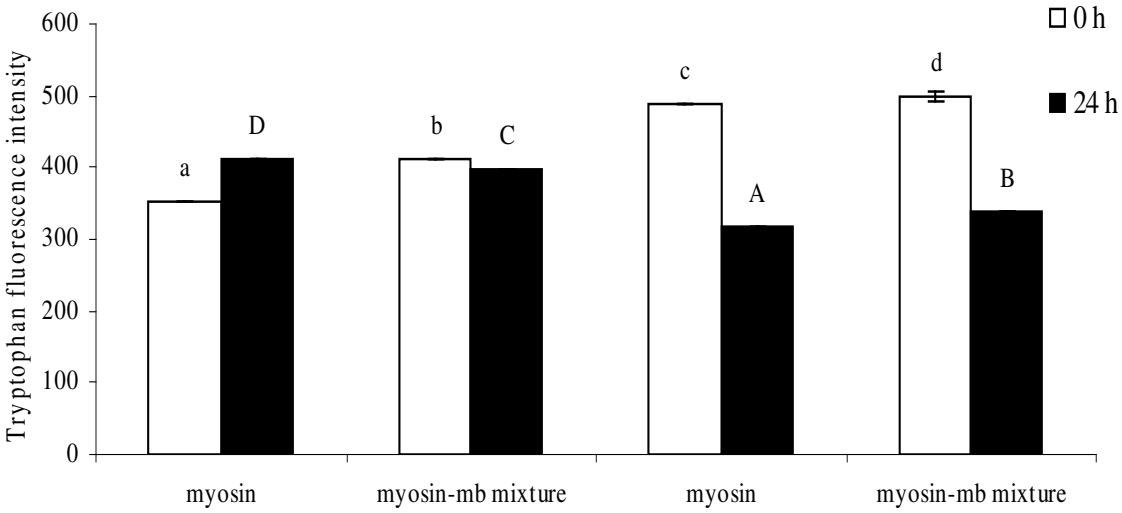


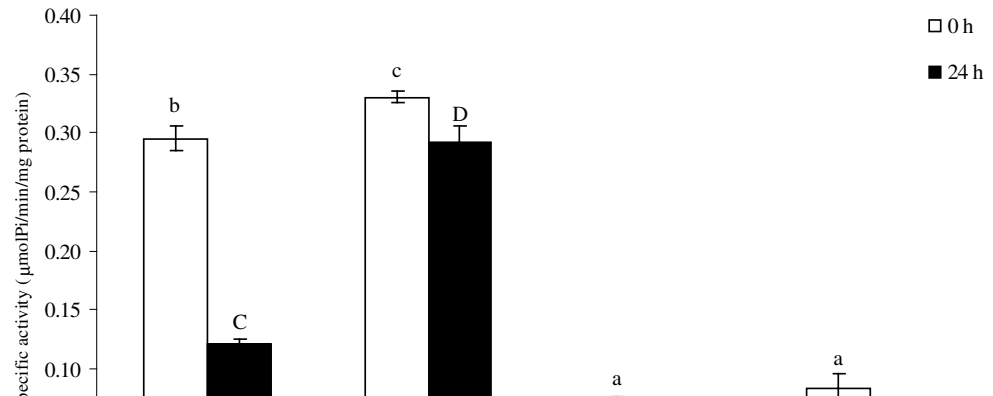
Figure 58. Tryptophan fluorescence intensity of the myoglobin and myoglobin-myosin mixtures during incubation at 4°C. Bars represent the standard deviation from triplicate determinations. Different letters or different letter case within the same incubation times indicate significant differences ($P<0.05$).

Among these residues, tyrosine and tryptophan are commonly found. An intrinsic tryptophan residue in the proteins indicated conformational changes of a tertiary structure (Chanthai *et al*., 1996). At 0 h of incubation, sardine myosin exhibited a greater fluorescent intensity than did tuna myosin ($P<0.05$). This might be due to the different amount of tryptophan resided in those myosin molecules. Furthermore, the degree of denaturation and/or the susceptibility of tuna and sardine myosin to denaturation might be different. The instantaneous increase in fluorescence intensity was observed when the myoglobin was mixed with both sardine and tuna myosins (0 h) ($P<0.05$). After 24 h of incubation, the fluorescence intensity of the control tuna myosin increased by approximately 17%, when compared with that found at 0 h of incubation. The increase in fluorescence intensity might be associated with the unfolding of tuna myosin with increasing incubation time. Conversely, the decreases in fluorescence intensity of tuna myosin-tuna myoglobin mixture, the control sardine myosin and sardine myosin-tuna myoglobin mixture were observed after 24 h of incubation ($P<0.05$). For the control sardine myosin incubated for 24 h, the conformational changes of protein might take place in the way, which hydrophobic interaction could be enhanced as shown by the lowered fluorescence intensity. Additionally, when tuna myoglobin was mixed with both tuna and sardine myosins, the fluorescence intensity decreased by 3.5 and 32.3%, respectively, after 24 h of incubation. From the result, the decrease in fluorescent intensity of sardine myosin-tuna myoglobin mixture was

about 10-fold greater than that of tuna myosin-tuna myoglobin mixture. The result suggested that hydrophobic residues in the mixture of tuna myoglobin and sardine myosin might be buried inside molecules after 24 h of incubation to a greater extent, compared with tuna myoglobin-tuna myosin mixture. This might be caused by the greater interaction between myoglobin and myosin, possibly via hydrophobic interaction of the former (Figure 56).

Effect of myoglobin-myosin interaction on Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activities of myosin and myosin-myoglobin mixture are shown in Figure 59. The initial Ca^{2+}-ATPase activity of tuna myosin was generally higher than that of sardine myosin (P<0.05). However, the Ca^{2+}-ATPase activity of both myosins decreased after 24 h of incubation (P<0.05). It was postulated that the denaturation of myosin molecules took place with increasing incubation time at 4°C. Ogawa *et al*.(1993) reported that myosin of tuna (*Thunnus obesus*) was more stable than sardine (*Sardinops melanostictus*) myosin as shown by the higher denaturation temperature (T_m) of the former. In the presence of tuna myoglobin, tuna myosin had the slight increase in Ca^{2+}-ATPase activity. For tuna myosin-tuna myoglobin mixture, the activity was increased after 24 h. The binding of myoglobin to myosin might induce the conformational changes, in the way which increased the Ca^{2+}-ATPase activity found at the head portion. Nevertheless, the decreases in Ca^{2+}-ATPase activity were noticeable in tuna myoglobin-sardine myosin after incubation (P<0.05). Thus, the conformational changes of both myoglobin and myosin affecting Ca^{2+}-ATPase activity were species specific as evidenced by different changes observed between tuna myoglobin-sardine myosin mixture and tuna myoglobin-tuna myosin mixture. Bound myoglobin might stabilize or reduce the myosin molecule from the denaturation over the storage period at 4°C. From the observation, the binding of myoglobin to myosin might take place over the period of incubation in different ways, depending upon the source of myosin added.



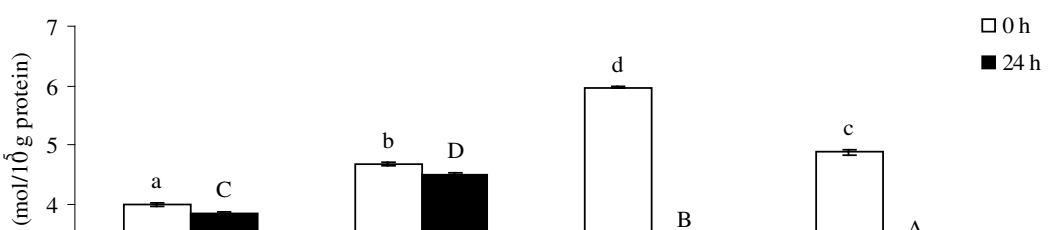


Figure 59. Ca^{2+}-ATPase activity of washed myosin and washed myoglobin-myosin mixtures during incubation at 4°C. Bars represent the standard deviation from triplicate determinations. Different letters or different letter case within the same incubation times indicate significant differences (P<0.05).

Effect of myoglobin-myosin interaction on reactive sulfhydryl (SH) content

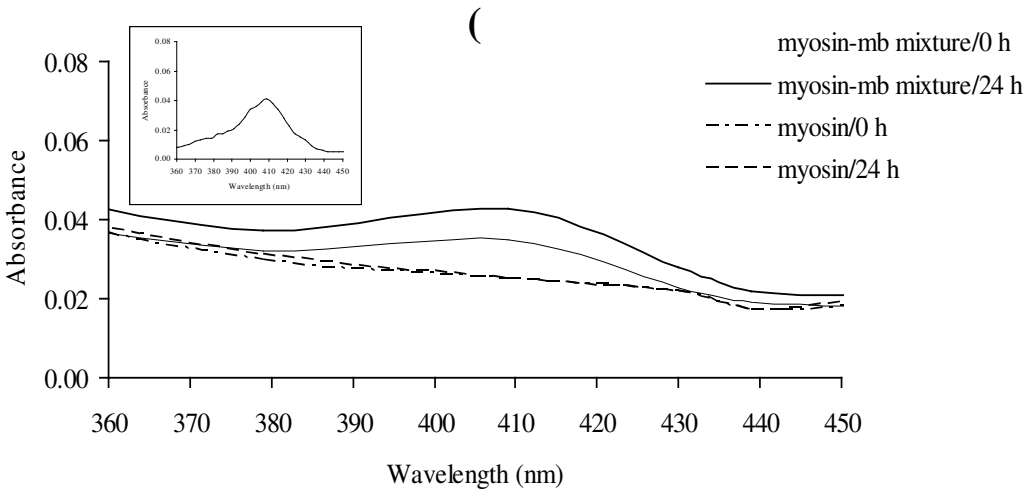
Reactive sulfhydryl (SH) contents of myosin and myosin-myoglobin mixture are shown in Figure 60. The initial SH group content of tuna myosin was lower than that of sardine myosin (P<0.05). However, the SH content of both myosins slightly decreased after 24 h of incubation (P<0.05). However, the decrease was more pronounced in sardine myosin. The decrease in SH group might be owing to the oxidation of SH to form disulfide cross-linkage within the myosin molecules. When tuna myoglobin was added into myoglobin, the SH content of tuna myosin-tuna myoglobin mixture increased, while the decrease in SH content was noticeable in tuna myoglobin-sardine myosin (P<0.05). The addition of tuna myoglobin seemed to supplement the SH content into the mixture. With increasing incubation time (24 h), disulfide bond could be formed between tuna myosin and tuna myoglobin as evidenced by the decrease in SH content in the mixture. From the result, the rate of decrease in SH content was found to vary, depending on the mixture.

Tuna Sardin

Figure 60. Reactive sulfhydryl content of washed myosin and washed myoglobin-myosin mixtures during incubation at 4°C. Bars represent the standard deviation from triplicate determinations. Different letters or different letter case within the same incubation times indicate significant differences (P<0.05).

Effect of myoglobin-myosin interaction on absorption spectra

The tuna myoglobin adducted to myosin in the mixtures was monitored by comparing the spectrum of the myoglobin-myosin mixture and the control myosin. Generally, the soret absorption band of tuna myoglobin was located in the blue region (350-450 nm) and its absorption peak was noticeable at 409 nm (Figure 61; inserted figure).



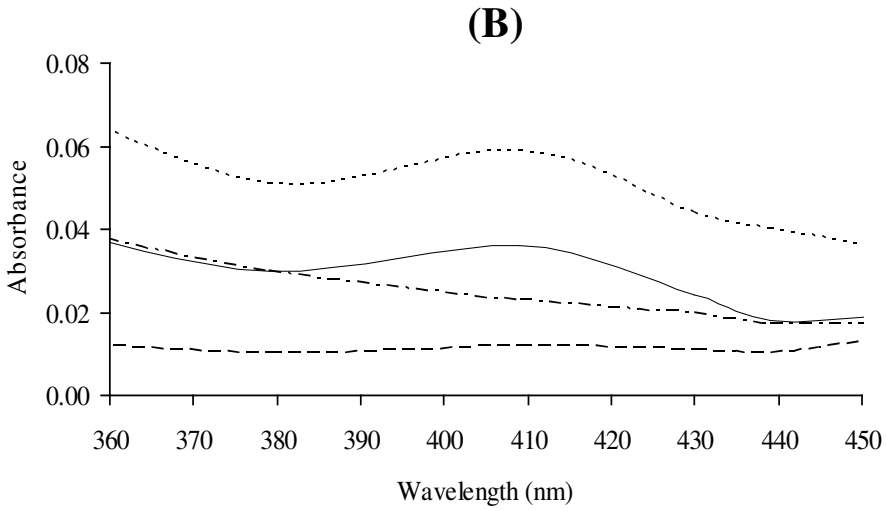


Figure 61. Absorption spectra of myosin and myoglobin-myosin mixtures during incubation at 4°C. (A), tuna myosin; (B), sardine myosin; inserted figure, typical absorption spectra of tuna myoglobin in soret region (350-450 nm).

The soret band of myoglobin-myosin and the control myosin is presented in Figure 61. For tuna myosin (Figure 61A), no absorption peak was observed after 0 and 24 h of incubation. The soret band was found in washed myoglobin-myosin mixture and the higher peak was found after 24 h incubation. This appearance of soret peak was in agreement with the increased bound myoglobin content found at 24 h (Figure 56). The tuna myoglobin bound to myosin was most likely associated with the higher absorption peak, especially after 24 h of incubation. For sardine myosin (Figure 61B), no absorption band of the control was also observed in both 0 and 24 h of incubation. However, the soret band with the higher peak was found in washed myoglobin-myosin mixture at 0 h than at 24 h. It was suggested that sardine myosin bound to myoglobin might exhibit the destabilization effect on myoglobin, resulting in the lowered absorption band observed. The disappearance of the soret absorption band indicated the destruction of the heme protein (Baron and Andersen, 2002)

10.5 Conclusions

Interaction between myosin and myoglobin led to the changes in reactive sulfhydryl content and tryptophan fluorescence intensity. The oxidation of oxymyoglobin was induced by this interaction. The losses in Ca^{2+}-ATPase activities of tuna and sardine myosins were enhanced in the presence of myoglobin. Appearance of soret band of washed myoglobin-myosin mixture confirmed the binding of myoglobin with myosin. Therefore, the extended *post-mortem* handling or storage of fish raw materials might be associated with the increased binding between myoglobin and myosin, leading to the difficulty in removal of myoglobin during washing process.