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Interaction between fish myoglobin and myosin *in vitro*

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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 found in the absence of myosin ($P < 0.05$). Oxymyoglobin underwent oxidation to a greater extent in the presence of tuna myosin than sardine myosin ($P < 0.05$). The interaction between fish myoglobin and myosin also caused 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.

Keywords: Fish; Myosin; Myoglobin; Interaction; Tuna; Sardine

1. Introduction

Dark fleshed-fish species have a high content of dark muscle, which comprises a considerable amount of lipids and sarcoplasmic proteins (Sikorski, Kolakowska, & Burt, 1990; Spinelli & Dassow, 1982). The presence of sarcoplasmic proteins and lipids in dark muscle is associated with its poorer gelation characteristics, compared with light muscle (Chen, 2002; Haard, Simpson, & Pan, 1994; Hultin & Kelleher, 2000; Ochiai, Ochiai, Hashimoto, & Watabe, 2001). Sarcoplasmic proteins have an adverse effect on the strength, deformability (Haard et al., 1994) and colour (Chaijan, Benjakul, Visessanguan, & Faustman, 2004; Chen, Chiu, & Huang, 1997) of fish myofibrillar protein gels. Myoglobin is the predominant pigment protein in the sarcoplasmic fraction of fish dark muscle (Hashimoto, Watabe, Kono, & Shiro, 1979) and contributes to the lowered whiteness of surimi gel (Chen, 2002). Generally, it is difficult to wash all the 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 the resulting surimi. However, heme proteins become less soluble as the fish undergo deterioration (Chaijan, Benjakul, Visessanguan, & Faustman, 2005; Chen, 2003). Denaturation of the myoglobin and/or myofibrillar proteins, before or during processing, can also cause their cross-linking, resulting in the discolouration of the surimi (Lanier, 2000). Recently, Chaijan, Benjakul, Visessanguan, Lee, and Faustman (accepted for publication) reported that the interaction between fish myoglobin and natural actomyosin was enhanced at higher ionic strength and higher temperature and the binding was augmented with increasing incubation times.

After capture, fish are normally kept in ice prior to unloading (Chaijan et al., 2005; Emilia & Santos-Yap, 1995). During this stage, discolouration of muscle and

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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.

2. Materials and methods

2.1. Chemicals

Sodium dodecyl sulfate (SDS), β-mercaptoethanol (βME), Triton X-100, Tris(hydroxymethyl)-aminomethane, sodium hydrosulfite, sodium phosphate, and potassium chloride were obtained from Sigma (St. Louis, MO, USA). Ammonium sulfate was purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Fish sample

Sardine (*Sardinella gibbosa*), with an average weight of 55–60 g, were caught from the 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 (*Thunnus albacares*), 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.

2.3. 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 of Triton X-100). The mixture was homogenised for 1 min using an IKA Labortechnik homogeniser (Selangor, Malaysia). The homogenate was centrifuged at $5000 \times g$ for 10 min at 4 °C, using a 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 $18\,000 \times g$ 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, pH 8.0, containing 1 mM EDTA, referred to as "starting buffer". The mixture was then dialysed 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.

2.4. Preparation of oxymyoglobin

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

2.5. Preparation of myosin

Myosin was extracted according to the method of Martone, Busconi, Folco, Trucco, and Sanchez (1986), as modified by Visessanguan, Ogawa, Nakai, and An, 2000. All steps were performed at 4 °C to minimise 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 $1000 \times g$ for 10 min. The pellet was suspended in five volumes of buffer B (0.45 M KCl, 5 mM βME, 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 $10\,000 \times g$ for 15 min. The 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 $12\,000 \times g$, resuspended gently with five volumes of buffer C (0.50 M KCl, 5 mM βME and 20 mM Tris–HCl, pH 7.5), and treated with three 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 $22\,000 \times g$ for 15 min. Myosin, recovered as a pellet, was used immediately or stored at −20 °C in 50% glycerol.

2.6. Study of 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) solubilised 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 three volumes of cold 10 mM phosphate buffer, pH 6.5, and centrifuged at $5000 \times g$ 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 analysed 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.

2.7. 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.

2.8. Determination of Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activities of myosin and washed myosin–myoglobin mixture were determined as described by Benjakul, Seymour, Morrissey, and An (1997). Myosin and myosin–myoglobin mixture were diluted to 2.5–8 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 $CaCl_2$. Deionised 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 $3500 \times g$ 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 protein/min. A blank solution was prepared by adding the chilled trichloroacetic acid prior to addition of ATP.

2.9. 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, Itoh, and Obataka (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 coefficient of $13\,600\ M^{-1}\ cm^{-1}$ and was expressed as mol/10^5 g protein.

2.10. 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, Neida, Ogawa, Tamiya, and Tsuchiya (1996).

2.11. Absorption spectra

The absorption spectra of myosin and the washed myosin–myoglobin mixture were determined using a ND-1000 Nanodrop spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE, USA), as described by Chaijan et al. (2005). The spectra were recorded from 350 to 450 nm at the scanning rate of 1000 nm/min, using 0.05 M potassium phosphate buffer, pH 6.5, containing 0.5 M KCl as a blank.

2.12. Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test (Steel & Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 10.0 for windows, SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Effect of myoglobin–myosin interaction on bound myoglobin content

The bound myoglobin content in myosin–myoglobin model systems is depicted in Fig. 1. Both sardine and tuna myosins bound to tuna myoglobin progressively during incubation at 4 °C for 24 h ($P < 0.05$). From this result, evidently, 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. This result suggested that both proteins could interact with 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

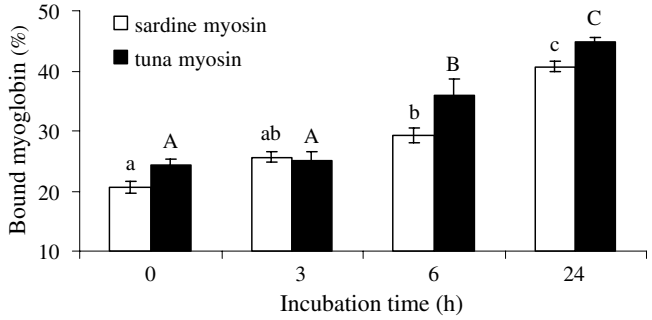

Fig. 1. Bound myoglobin content (%) in fish myoglobin–myosin mixtures during incubation at 4 °C. Bars represent the standard deviation from triplicate determinations. Different letters or different letter cases within the same fish myosins indicate significant differences ($P < 0.05$).

and myoglobin might occur in a way which enhanced the interaction between those proteins. Generally, tuna myoglobin was adducted with tuna myosin to a greater extent, than was sardine myosin. From this result, the interaction between fish myoglobin and myosin appeared to be species-specific and increased with increasing incubation time.

3.2. 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 ($P < 0.05$) (Fig. 2). Generally, the oxidation of fish myoglobin was more pronounced with increasing iced storage time (Chaijan et al., 2005; Chen, 2003). The formation of metmyoglobin in the presence of myosin was generally greater than that without myosin. In the presence of tuna myosin, myoglobin underwent the oxidation more intensively, compared with the system containing sardine myosin. 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

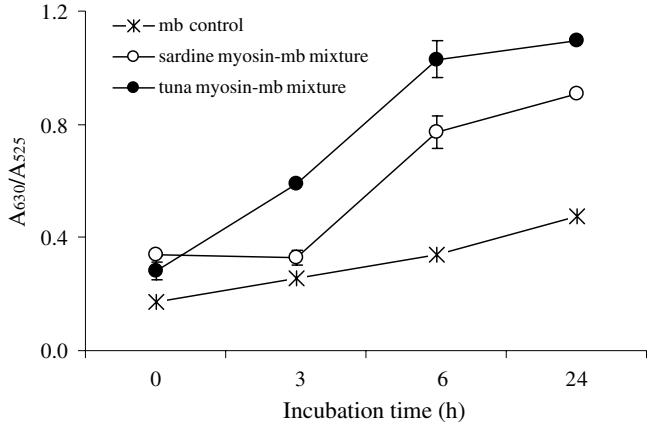

Fig. 2. Metmyoglobin formation (A_{630}/A_{525}) in the myoglobin and myoglobin–myosin mixtures during incubation at 4 °C. mb: myoglobin. Bars represent the standard deviation from triplicate determinations.

1.9-fold higher than that observed in the control myoglobin. This result suggested that the conformational changes of globin could occur easily when myoglobin was adducted with myosin, especially from the same fish species. As a result, it might favour the oxidation of the heme molecule. Xiong (1997) reported that the globin portion of myoglobin played an important role in protecting heme against oxidation. When the conformation of globin is altered, even minimally, particularly with extended storage, myoglobin is more prone to oxidation. Eder (1996) reported that myoglobin was susceptible to oxidation when it lost the native conformation.

3.3. Effect of myoglobin–myosin interaction on tryptophan fluorescence intensity

The tryptophan fluorescence intensity of myosin and myosin–myoglobin mixture during incubation at 4 °C for 24 h is shown in Fig. 3. Copeland (1994) pointed out that when a protein unfolds, amino acid residues, which are buried in the nonpolar interior of the protein, become exposed to the polar aqueous solvent. Among these residues, tyrosine and tryptophan are commonly found. The fluorescence spectroscopy of these amino acids offers a very powerful tool to monitor changes in protein structure (Copeland, 1994). Delocalisation of intrinsic tryptophan residue in the proteins indicated conformational changes of a tertiary structure (Chanthai et al., 1996). Generally, fish myoglobins contained one or two tryptophan residues (Chanthai et al., 1996). At 0 h of incubation, sardine myosin exhibited a greater fluorescence intensity than did tuna myosin ($P < 0.05$). This might be due to the different amount of tryptophan resident in those myosin molecules. Watts, Rice, and Brown (1980) reported that yellowfin tuna comprised one tryptophan residue. Furthermore, the degree of denaturation and/or the susceptibility of tuna and sardine myosin to denaturation might be different. 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, in 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 this result, the decrease in fluorescence intensity of sardine myosin–tuna myoglobin

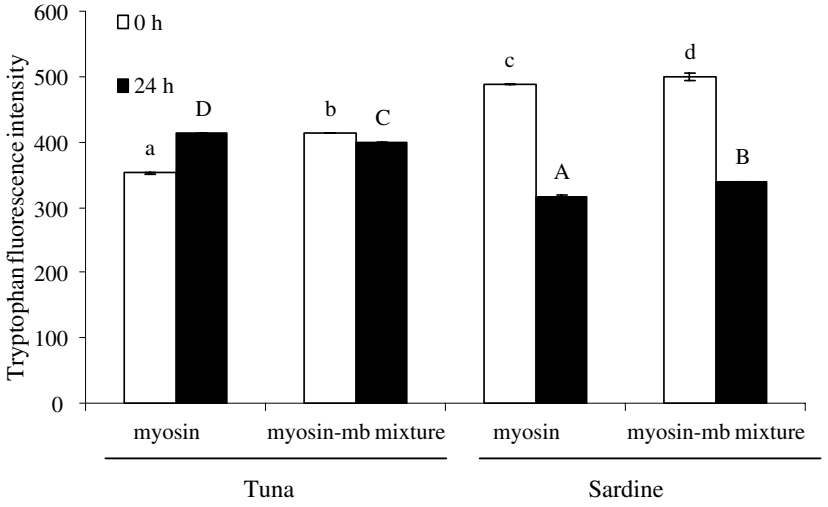

Fig. 3. Tryptophan fluorescence intensity of the myoglobin and myoglobin–myosin mixtures during incubation at 4 °C. mb: myoglobin. Bars represent the standard deviation from triplicate determinations. Different letters or different letter cases within the same incubation times indicate significant differences ($P < 0.05$).

mixture was about 10-fold greater than that of tuna myosin–tuna myoglobin mixture. Evidently 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, than in the tuna myoglobin–tuna myosin mixture. This might be caused by the greater interaction between myoglobin and myosin, possibly via hydrophobic interaction of the former mixture (Fig. 1).

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

Ca^{2+}-ATPase activities of myosin and myosin–myoglobin mixture are shown in Fig. 4. Generally, myofibrillar ATPase activities have been widely used as a measure of actomyosin integrity (Roura, Monteccia, Goldemberg, Trucco, & Crupkin, 1990). The loss of enzymatic activity reflects the extent of damage and alteration of the protein structure in the muscle system (Jiang & Lee, 1985). 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 activities of both myosins decreased after 24 h of incubation ($P < 0.05$). Apparently the denaturation of myosin molecules took place with increasing incubation time at 4 °C. Ogawa, Ehara, Tamiya, and Tsuchiya (1993) reported that myosin of tuna (*Thunnus obesus*) was more stable than was 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 a slight increase in Ca^{2+}-ATPase activity. For the tuna

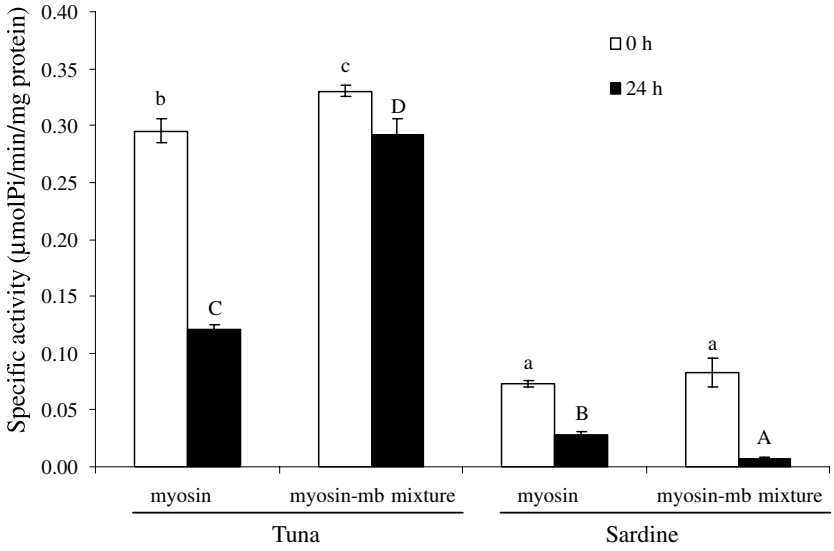

Fig. 4. Ca^{2+}-ATPase activities of washed myosin and washed myoglobin–myosin mixtures during incubation at 4 °C. mb: myoglobin. Bars represent the standard deviation from triplicate determinations. Different letters or different letter cases within the same incubation times indicate significant differences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

myosin–tuna myoglobin mixture, the activity was increased after 24 h. The binding of myoglobin to myosin might induce conformational changes, in a way which increased the Ca^{2+}-ATPase activity found at the head portion. Nevertheless, 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 affected Ca^{2+}-ATPase activity and were governed by the sources of proteins. Bound myoglobin might stabilise or reduce the myosin molecule from the denaturation over the storage period at 4 °C. From this observation, the binding of myoglobin to myosin might take place over the period of incubation in different ways, depending on the source of myosin added.

3.5. Effect of myoglobin–myosin interaction on reactive sulfhydryl (SH) content

Reactive sulfhydryl (SH) contents of myosin and myosin–myoglobin mixture are shown in Fig. 5. The initial SH group content of tuna myosin was lower than that of sardine myosin ($P < 0.05$). However, the SH contents 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 the SH group might be due to the oxidation of SH to form disulfide cross-linkages within the myosin molecules. Ishioroshi, Samejima, and Yasui (1982) reported that the oxidation of SH groups on the head region of heavy-meromyosin (HMM) was involved in the aggregation and network formation of myosin. When tuna myoglobin was added to myosin, the SH content of tuna myosin–tuna myoglobin mixture increased, while a decrease in SH content was noticeable in the tuna myoglobin–sardine myosin ($P < 0.05$). The addition of tuna myoglobin seemed to supplement the SH content into the mixture. Brown (1961) reported that some fish myoglobins contain a cysteine residue, which could influence the susceptibility of myoglobin to oxidation. With increasing incubation time (24 h), disulfide bonds could be formed between tuna myosin and tuna myoglobin, as evidenced by the decrease in SH content in the mixture. From this result, the rate of decrease in SH content was found to vary, depending on the mixture.

3.6. 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 (Fig. 6; inset figure). The soret peak of sardine (*Sardinella gibbosa*) oxymyoglobin was found at 405 nm (Chaijan, Benjakul, Visessanguan, & Faustman, 2007). Nevertheless, Swatland (1989) reported that the soret bands of deoxymyoglobin, oxymyoglobin and metmyoglobin in meat were 434, 416 and 410 nm, respectively. The spectra of myoglobin derivatives vary considerably, depending on the state of iron and the molecule to which it is bound (Millar, Moss, & Stevenson, 1996). The soret bands of myoglobin–myosin and the control myosin are presented in Fig. 6. For tuna myosin (Fig. 6a), 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 of incubation. This appearance of the soret peak was in agreement with the increased bound myoglobin content found at 24 h (Fig. 1). The tuna myoglobin bound to myosin was most likely associated with the higher absorption peak, especially after 24 h of incubation. For sardine myosin (Fig. 6b), no absorption band of the control was observed after 0 and 24 h of incubation. However, the soret band was found with a higher peak in washed myoglobin–

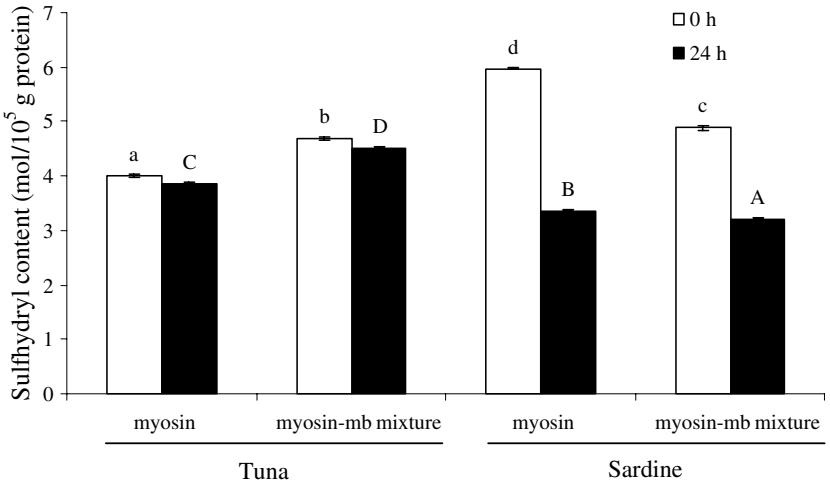

Fig. 5. Reactive sulfhydryl contents of washed myosin and washed myoglobin–myosin mixtures during incubation at 4 °C. mb: myoglobin. Bars represent the standard deviation from triplicate determinations. Different letters or different letter cases within the same incubation times indicate significant differences ($P < 0.05$).

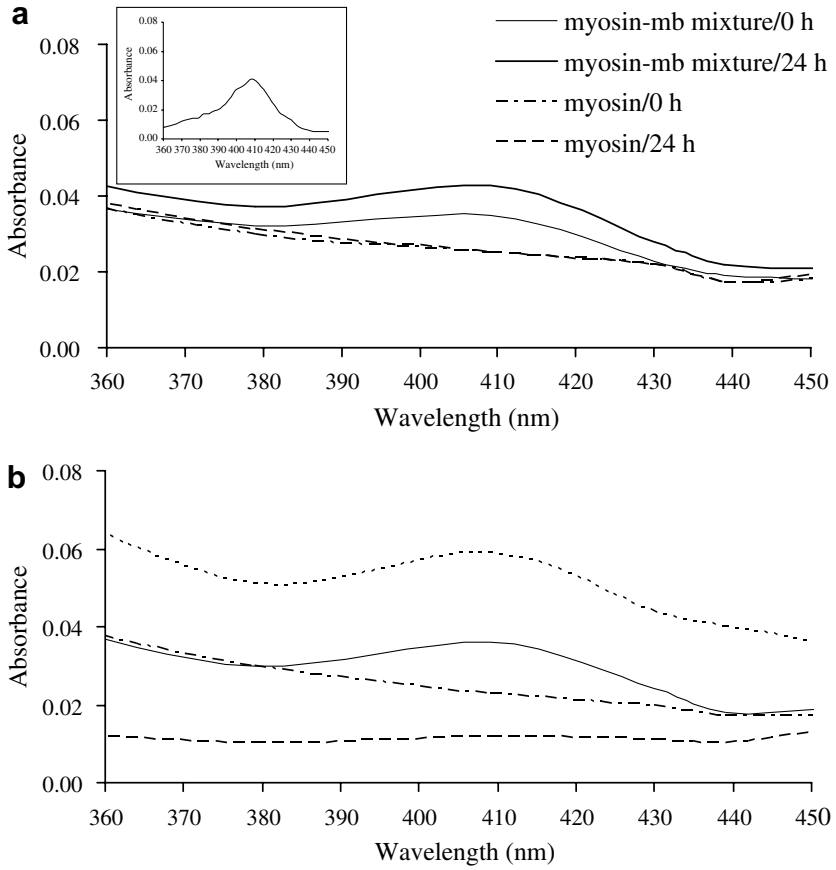

Fig. 6. Absorption spectra of myosin and myoglobin–myosin mixtures during incubation at 4 °C. (a) Tuna myosin; (b) sardine myosin; inset figure, typical absorption spectrum of tuna myoglobin in soret region (350–450 nm). mb: myoglobin.

myosin mixture at 0 h than at 24 h. Sardine myosin, bound to myoglobin, might have a destabilisation effect on myoglobin, resulting in the lowered absorption band observed. The disappearance of the soret absorption band indicated destruction of the heme protein (Baron & Andersen, 2002).

4. Conclusion

Interaction between fish 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 a 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 difficulty in removal of myoglobin during the washing process.

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