CHAPTER 6

CHARACTERIZATION OF MYOGLOBIN FROM SARDINE (*SARDINELLA GIBBOSA*) DARK MUSCLE

6.1 Abstract

Myoglobin from the dark muscle of sardine (*Sardinella gibbosa*) with the molecular weight of 15.3 kDa was isolated and characterized. The different myoglobin derivatives exhibited varying thermal unfolding characteristics. Deoxymyoglobin showed a single distinct endothermic peak at 74.5°C, whereas two transition temperatures were noticeable for oxymyoglobin (64.5°C and 78.4°C) and metmyoglobin (59.0°C and 76.0°C). The spectrum of deoxymyoglobin and oxymyoglobin had absorption bands at 739, 630, 575, 500 and 405 nm, while the disappearance of the peak at 575 nm was found in the spectrum of metmyoglobin. The soret peak of all derivatives was noticeable at 405 nm. The autoxidation of myoglobin became greater at very acidic or alkaline conditions as evidenced by the formation of metmyoglobin, the changes in tryptophan fluorescence intensity as well as the disappearance of soret absorption. The higher temperature, particularly above 40°C, and the longer incubation time induced the higher metmyoglobin formation as well as the conformational changes.

6.2 Introduction

Myoglobin is a globular heme protein localized in red muscle fibers. Myoglobin concentration generally depends on species, breed, sex and age of animal, training and nature of nutrition, muscular activity, oxygen availability, blood circulation and muscle type as well as the way the meat is treated (Postnikova *et al*., 1999; Livingston and Brown, 1981; Giddings, 1974). Myoglobin has been known to be a major contributor to the color of muscle, depending upon its derivatives and concentration (Postnikova *et al*., 1999; Faustman *et al*., 1992). The stability of

myoglobin also affects the color of meat (Chen, 2003; Chanthai *et al*., 1998; Suzuki and Kisamori, 1984).

The use of under-utilized small pelagic fish species, such as sardine and mackerel, for surimi production is limited, mainly due to the large quantity of lipids and myoglobin in the muscle tissue. Chaijan *et al*. (2004) reported that lipid and myoglobin contents were higher in dark muscle than in ordinary muscle of both sardine and mackerel, and higher contents of both constituents were found in sardine muscle than mackerel muscle. Myoglobin and hemoglobin play an essential role in the whiteness of surimi (Chen, 2002). Hemoglobin is lost rather easily during handling and storage, while myoglobin is retained by the muscle intracellular structure (Livingston and Brown, 1981). Therefore, color changes in meat are most likely due to the reaction of myoglobin with other muscle components, especially myofibrillar proteins (Hanan and Shaklai, 1995). Since the abundant dark muscle is difficult to remove with a meat separator for red-fleshed fish (Ochiai *et al*., 2001), changes in myoglobin may be associated with the discoloration of resulting surimi. During the handling and storage of fish, a number of biochemical, chemical and microbiological changes also occur and are associated with the discoloration (Pacheco-Aguilar *et al*., 2000; O'Grady *et al*., 2001; Faustman *et al*., 1992). Discoloration of tuna during frozen storage is caused by the formation of metmyoglobin (Haard, 1992). This phenomenon can be influenced by many factors such as pH, temperature, ionic strength and oxygen consumption reaction (Renerre and Labas, 1987). Metmyoglobin formation is positively correlated with lipid oxidation (Lee *et al*., 2003a; Chan *et al*., 1997). Recently, Chaijan *et al*. (2005a) reported that prolonged iced storage decreased the myoglobin extracting efficiency in sardine and mackerel muscle due to the insolubility of myoglobin caused by the oxidation of myoglobin to form metmyoglobin. This change might be related to the intrinsic and extrinsic factors determining the myoglobin molecules. However, information regarding the characteristics and properties of myoglobin from sardine, which has been paid increasing attention as raw material for surimi processing, is scarce. The objective of this investigation was to partially purify and characterize the myoglobin from sardine dark muscle.

6.3 Materials and Methods

Chemicals

Sodium dodecyl sulfate (SDS), dithiothreitol (DTT), β-mercaptoethanol (βME) and Triton X-100 were purchased from Sigma (St. Louise, MO, USA). Acrylamide, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), bis-acrylamide, disodium hydrogen phosphate and sodium dihydrogen phosphate were purchased from Fluka (Buchs, Switzerland). Sodium dithionite was obtained from Riedel (Seeize, Germany). Sodium chloride and Tris (hydroxymethyl)-aminomethane were procured from Merck (Damstadt, Germany). All chemicals were of analytical grade.

Fish samples

Sardine (*Sardinella gibbosa*) with an average weight of 55-60 g was caught from Songkhla-Pattani Coast along the Gulf of Thailand in March, 2005. 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 1-2 h. The fish were immediately washed, filleted and manually excised. The dark muscles were collected and used for myoglobin extraction.

Extraction and purification of myoglobin from sardine muscle

Extraction and purification of myoglobin was performed according to the methods of Trout and Gutzke (1996) with a slight modification. The dark 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). After centrifugation at 9,600×*g* for 10 min at 4°C using the RC-5B plus centrifuge (Sorvall, Norwalk, CT, USA), the supernatant was filtered through a Whatman No. 4 filter paper. The filtrate was then subjected to ammonium

sulfate fractionation. The precipitate obtained with 65-100% saturation was dissolved in a minimal volume of cold 5 mM Tris-HCl buffer, pH 8.5, which was referred to as starting buffer. The mixture was then dialyzed against 10 volumes of same buffer with 20 changes at 4°C. The dialysate was immediately applied to a Sephadex G-75 column (2.6 × 70 cm; Amersham Bioscience, Uppsala, Sweden) which was equilibrated with the starting buffer. The separation was conducted using the starting buffer at a flow rate of 0.5 ml/min and 3-ml fractions were collected. The fractions containing myoglobin were combined and further purified by ion-exchange chromatography on a HiTrap DEAE FF column (prepacked 5 ml; Amersham Biosciences, Uppsala, Sweden). After the column was washed with starting buffer, the elution was performed with a linear gradient of 0-0.5 M NaCl in starting buffer at a flow rate of 1 ml/min. Fractions of 3 ml were collected and those with high content of myoglobin were pooled.

During purification, the fractions obtained were measured at 280 nm and 540 nm using a UV-1601 spectrophotometer (Shimadzu, Japan). The fractions with the high absorbance at 540 nm were also subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Determination of molecular weight

The molecular weight of myoglobin was determined by SDS-PAGE using 4% stacking gel and 17.5% separating gel according to the method of Laemmli (1970). The electric current for each gel was maintained at 15 mA using a Mini Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie brilliant blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% methanol (v/v) and 7.5% (v/v) acetic acid, followed by 5% methanol (v/v) and 7.5% (v/v) acetic acid. The myoglobin separated on SDS-PAGE was estimated for its molecular weight by plotting the logarithm of molecular weight of the protein standards against relative mobility. The low-molecular-weight-protein standards [albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), □-lactalbumin (14.2 kDa) and aprotinin (6.5 kDa)] were used.

Preparation of myoglobin derivatives

Derivatives of sardine myoglobin were prepared according to the method of Millar *et al*. (1996) with some modifications. An aliquot (5 ml) of purified myoglobin was converted to deoxymyoglobin by the addition of 50 mg of sodium dithionite. Oxymyoglobin was obtained by gently agitating deoxymyoglobin solution with purging of O_2 gas at a flow rate of 3 ml/min for 1 h. Metmyoglobin was prepared by adding 50 mg of potassium ferricyanide to 5 ml of the purified myoglobin solution. The sodium dithionite and potassium ferricynide were removed by dialysis the sample against 10 volumes of cold 50 mM phosphate buffer, pH 7.0 with 20 changes. The protein concentration of each myoglobin derivative was adjusted to 0.2 mg/ml with cold 50 mM phosphate buffer, pH 7.0. All the procedures were carried out at 4°C.

Absorption spectra

The absorption spectra of myoglobin derivatives were taken using a V-530 UV/VIS double beam spectrophotometer (Jasco, Tokyo, Japan) as described by Chaijan *et al*. (2005a). The spectra were recorded from 350 to 750 nm at the scanning rate of 1,000 nm/min using 50 mM phosphate buffer, pH 7.0 as a blank.

Differential scanning calorimetry (DSC)

The DSC thermograms of myoglobin derivatives were determined according to the method of Chen (2003) using Perkin Elmer Differential Scanning Colorimeter (DSC) (Model DSC-7, Norwalk, CT, USA.). Temperature calibration was performed using the Indium thermogram. The specimen (about 12 mg) was accurately weighed, placed into an aluminium pan and sealed. The scanning was performed at a heating rate of 2°C/min over the temperature range of 20-100°C. The ice water was used as a cooling medium and the system was equilibrated at

20°C for 5 min prior to the scan. The empty aluminium pan was used as a reference. The temperature at each peak maximum was recorded as the transition temperature (T_m).

Protein determination

Protein concentration of myoglobin solution was determined by the Lowry method (Lowry *et al*., 1951) using bovine serum albumin as standard.

Effect of pH and temperature on the stability of sardine myoglobin

For the pH stability study, the portions of oxymyoglobin solution were dialyzed against 10 volumes of cold 50 mM phosphate buffers with various pH values (pHs 4, 5, 6, 7, 8, 9, 10, 11, and 12) for 15 h according to the method of Chow *et al*. (1985). Protein concentrations of myoglobin solution were determined by the Lowry method (Lowry *et al*., 1951) and adjusted to 0.2 mg/ml with the respective buffers.

Thermal stability of myoglobin was studied. Three ml of oxymyoglobin in 50 mM phosphate buffer, pH 7.0 (0.2 mg protein/ml) was pipetted into a test tube and subjected to the incubation at 20, 30, 40, 50, 60 and 70°C in the temperature-controlled-waterbath (Memmert, Germany) for 10 and 30 min. The treated samples were suddenly cooled in iced water. The myoglobin solution (0°C) was used as the control. The metmyoglobin content, tryptophan fluorescence and absorption spectra of resulting myoglobins were determined.

Tryptophan fluorescence measurement

Tryptophan fluorescence measurement of myoglobin solutions was performed with a RF-1501 spectrofluorometer (Shimadzu, Kyoto, Japan) at an excitation wavelength of 280 nm and an emission wavelength of 325 nm according to the method of Chanthai *et al*. (1996).

Metmyoglobin content determination

The analysis of metmyoglobin content was performed as described by Lee *et al*. (1999). The sample was subjected to the absorbance measurement at 700, 572, and 525 nm. The percentage of metmyoglobin was calculated using the following equation (Krzywicki, 1982):

$$\%\text{Metmyoglobin} = \left\{ 1.395 - \left[(A_{572} - A_{700}) / (A_{525} - A_{700}) \right] \right\} \times 100.$$

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).

6.4 Results and Discussion

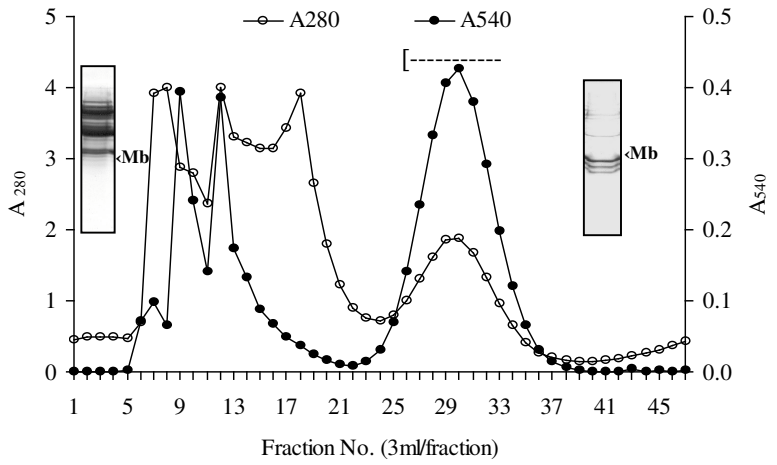
Purification of myoglobin from sardine dark muscle

A crude myoglobin extract was subjected to ammonium sulfate fractionation and the precipitate obtained at 65-100% saturation was then further purified by gel filtration using the Sephadex G-75 column. Two major peaks exhibiting A_{540} were observed (Figure 22A). Based on the molecular weight determined by SDS-PAGE, the first peak with a brown color might contain hemoglobin and other high-molecular-weight proteins. The second peak with the same color was most likely myoglobin, based on its molecular weight (15.3 kDa). To purify myoglobin obtained from previous step, an ion-exchange chromatography was applied using a HiTrap DEAE FF coloum. After washing and eluting with a 0-0.5 M NaCl linear gredient, one major peak with the brown color was eluted at 0.15-0.25 M NaCl (Figure 22B) and considered to be myoglobin according to its molecular weight measured by SDS-PAGE.

The SDS-PAGE patterns of sardine myoglobin extract during purification process are shown in Figure 23. Sardine dark muscle extracted with 10 mM Tris-HCl, pH 8.0 containing 1 mM EDTA in the presence of 25 g/l Triton X-100 contained a large number of

proteins. Protein with a molecular weight of 47 kDa was found to be dominant (Figure 23, lane 1). After ammonium sulfate precipitation (65-100% saturation), proteins with the molecular weight greater than 70 kDa were removed, while proteins with molecular weight of 64, 47 and 42 kDa became predominant. Coincidentally, protein bands with the molecular weight of 15.3 and 13 kDa appeared (Figure 23, lane 2). After gel filtration, proteins with molecular weight higher than 15.3 kDa were completely removed and protein band with the molecular weight of 15.3 kDa became intense. However, the band with molecular weight of 6.5 kDa was noticeable (Figure 23, lane 3). After subjecting to DEAE chromatography, protein with the molecular weight of 6.5 kDa disappeared and protein with molecular weight of 15.3 kDa, which was most likely myoglobin, became predominant (Figure 23, lane 4).

(A)



(B)

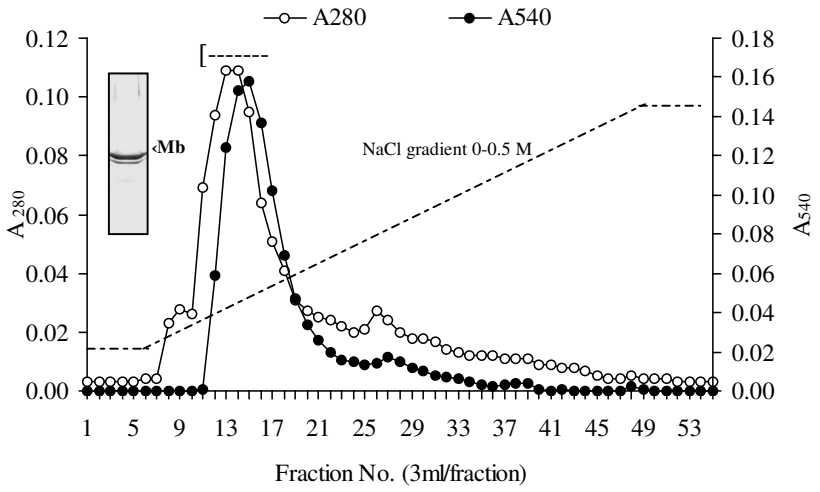


Figure 22. Elution profile of sardine myoglobin on the Sephadex G-75 (A) and Hitrap DEAE FF ion-exchange chromatography (B).

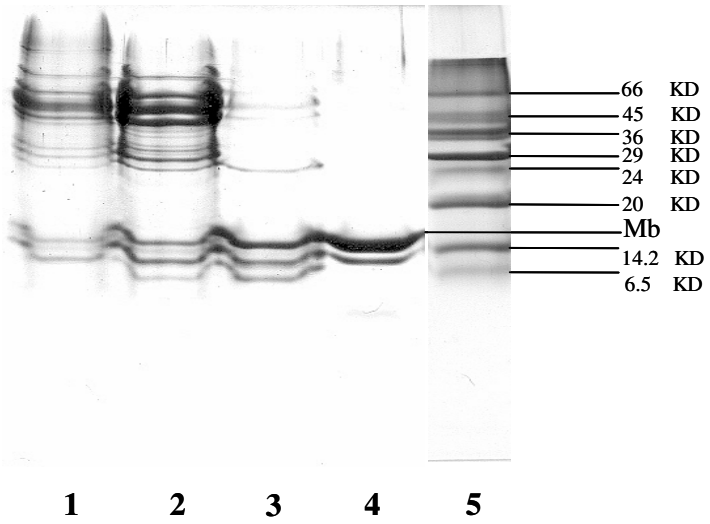


Figure 23. SDS-PAGE pattern of partially purified myoglobin from sardine dark muscle. Lane 1, crude extract; lane 2, ammonium sulfate precipitated fraction; lane 3, Sephadex G-75 fraction; lane 4, HiTrap DEAE FF fraction; lane 5, low-molecular- weight standard. Mb, sardine myoglobin.

The densitometric analysis showed that the myoglobin band represented about 92% of the total protein in pooled DEAE fraction. The result suggested that the increased purity was obtained after 3 steps of purification. From the result, the molecular weight of myoglobin was determined to be 15.3 kDa. The molecular weight of sardine myoglobin was similar to those of yellowfin tuna, bigeye tuna and milkfish, which were 16,000 daltons (Fosmire and Brown, 1976), 15,540 daltons (Ueki and Ochiai, 2004) and 15,900 daltons (Chen and Chow, 2001), respectively. Myoglobin from mackerel and another species of sardine (*Sadinops melanosticta*) had the molecular weight of 14,900 and 14,600 daltons, respectively (Yamaguchi *et al*., 1979). Furthermore, 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. Renerre *et al*. (1992) reported that bovine myoglobin had the molecular weight of about 17,000 daltons. It was noted that the molecular weight of fish myoglobin is generally smaller than that of mammalian myoglobin.

Differential scanning calorimetry (DSC) of different myoglobin derivatives

The DSC thermograms of different myoglobin derivatives from sardine dark muscle are shown in Figure 24. Different myoglobin derivatives showed varying thermal unfolding characteristics. From the result, a single distinct endothermic peak was observed at 74.5°C for deoxymyoglobin. Oxymyoglobin revealed two transition temperatures (T_m) at 65.4°C and 78.4°C. T_m of 59.0°C and 76.0°C were observed for metmyoglobin (Figure 24). All myoglobin derivatives might be different in either compactness of the globin fold or structural configuration. Oxymyoglobin and metmyoglobin from sardine dark muscle therefore exhibited structural unfolding with a multi-state transition. Chanthai *et al*. (1996) reported that most fish myoglobin had two T_m, reflecting multiple states of structural unfolding. However, myoglobin from some fish such as sea perch and tilapia exhibited a single endothermic peak (Chen *et al*., 2004). From the result, all derivatives had the endothermic peak in the some temperature range (74.5-76.0°C) though the different peak area was noticeable. This result suggested that oxymyoglobin and metmyoglobin might be transformed to deoxymyoglobin during dialysis process in which sodium dithionite and potassium ferricynide were removed. As a consequence, the deoxy form might be regained to some extent as shown by the remaining peak at 74.8°C and 76.0°C for oxy and met form, respectively. Peak area tended to decrease when the myoblobin molecule was occupied by oxygen molecule. When myoglobin was oxidized to metmyoglobin, this peak area also decreased markedly. From the result, the lowest T_m and lowest area peak were found in metmyoglobin, suggesting the lowest thermostability of this form. It can be inferred that thermal transition of sardine myoglobin depended on its forms. The heme moiety in metmyoglobin might be loosely fixed (Eder, 1996). As a consequence, the reversible dissociation into heme and apoproteins could occur easily (Eder, 1996). Unlike oxygen which can only bind to the ferrous form of heme pigments, water can bind only the ferric form (Eder, 1996). From the result, the T_m of myoglobin shifted to the lower temperature and the peak area decreased markedly when the myoglobin was predominantly in the ferric state (metmyoglobin).

The result suggested that the buried water molecule contributed to the conformational stability of myoglobin. Hinz *et al*. (1993) reported that the T_m of glucose oxidase decreased with the increase water content in their molecules. Kendrew *et al*. (1960) reported that the interior of the myoglobin molecule was almost entirely made up

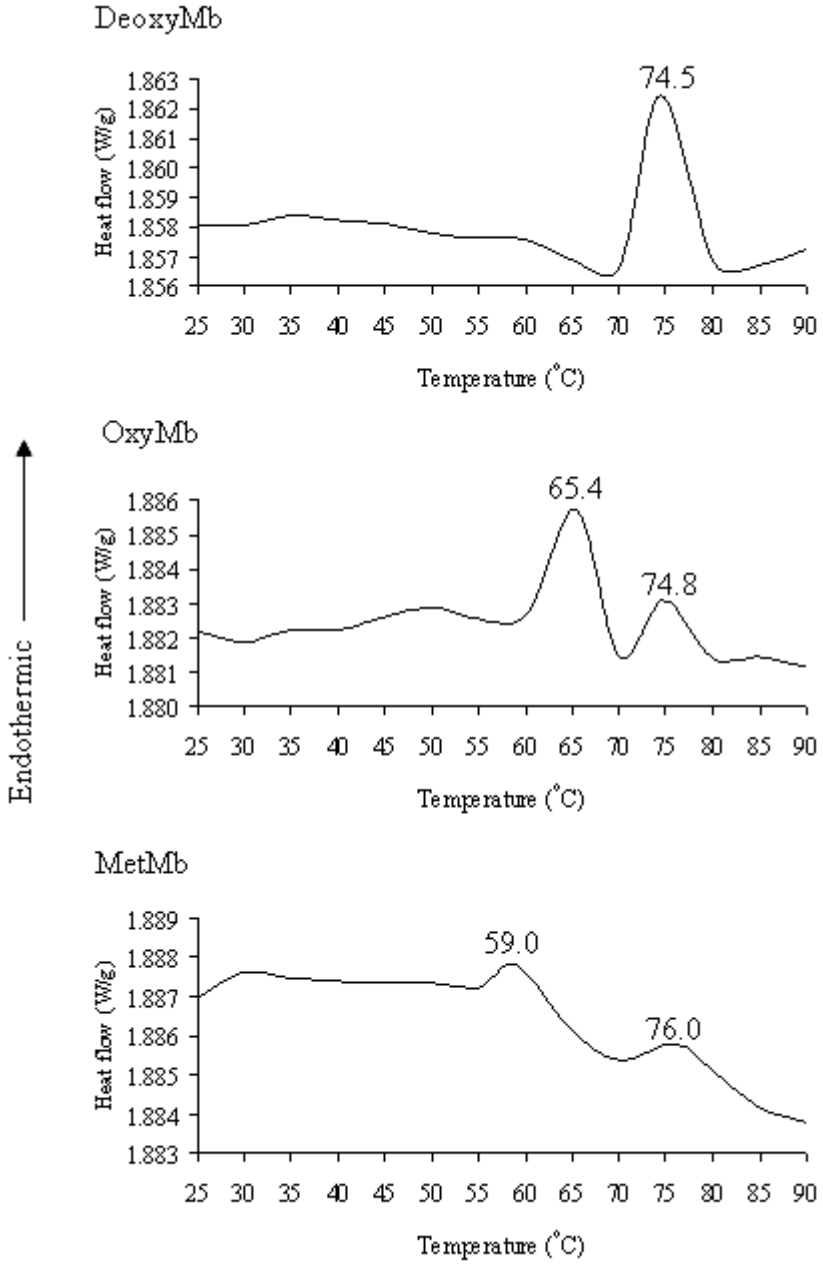


Figure 24. Differential scanning calorimetry thermograms of different sardine myoglobin derivatives.

of non-polar residues, generally close-packed and in van der Waals contact with their neighbours. Chen (2003) reported that T_m of myoglobin shifted to the lower temperature when metmyoglobin formation increased during the refrigerated storage of mackerel myoglobins. Therefore, deoxymyoglobin was the most heat-stable form, followed by oxymyoglobin and metmyoglobin, respectively. This observation was in agreement with Machilik (1965) who reported that the resistance to denaturation by heat was greatest in deoxymyoglobin, followed by oxymyoglobin, and was lowest for metmyoglobin.

Absorption spectra of different myoglobin derivatives

The spectra of myoglobin derivatives from sardine dark muscle in the ranges of 350-750 nm are shown in Figure 25. The spectra of deoxymyoglobin and oxymyoglobin had absorption bands at 739, 630, 575, 500 and 405 nm. The absorbance spectrum of metmyoglobin was distinguished from those of deoxymyoglobin and oxymyoglobin as shown by the disappearance of the peak at 575 nm (Figure 25A). Young and West (1996) reported that the reduced myoglobin (Fe^{2+}; deoxy and oxy-form) showed the isobestic point at 572 nm. The spectrum of sperm whale myoglobin had the absorption band at 556 and 434 nm for deoxy form, 543, 581 and 418 nm for oxy form and 635, 505 and 409.5 for met form, respectively (Govindarajan, 1979). The strong absorption of all myoglobin derivatives was located in the blue region (350-450 nm) or soret band (Figure 25B). The soret peak of all myoglobin derivatives studied was found at 405 nm. Nevertheless, Swatland (1989) reported that the soret bands of deoxymyoglobin, oxymyoglobin and metmyoglobin in meat were 434, 416 and 410 nm,

respectively. Similar peak height was observed between oxymyoglobin, and deoxymyoglobin, whereas metmyoglobin showed the lowest peak height (Figure 25A, B). From the result, the oxidation caused the changes in absorption characteristic of myoglobin as evidenced by the loss of the absorbance band at 575 nm and the lowered absorbance in the soret region. Millar *et al.* (1996) reported that the spectra of myoglobin derivatives vary considerably depending on the state of iron and the molecule to which it is bound.

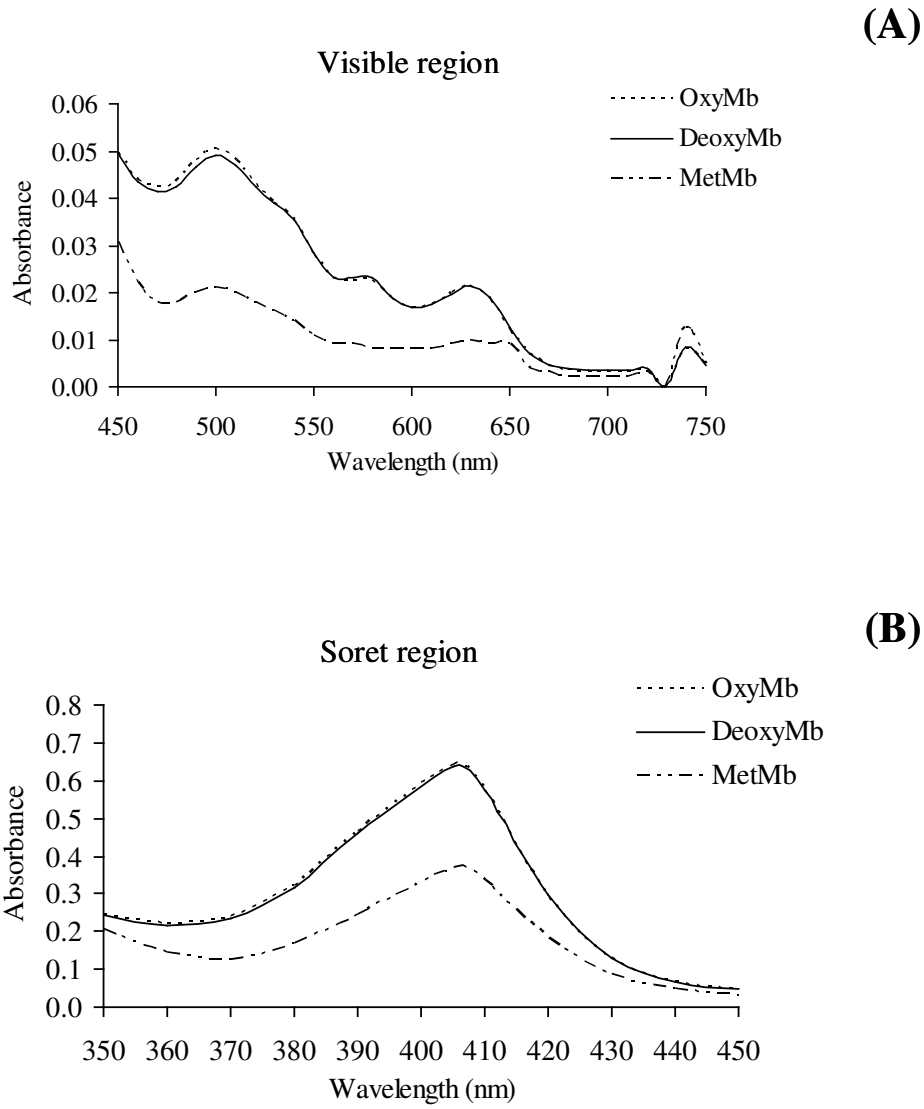


Figure 25. The absorption spectra of different sardine myoglobin derivatives in the visible region (450-750 nm) (A) and soret region (350-450) (B).

Effect of pH on myoglobin stability

The metmyoglobin formation at different pHs is shown in Figure 26A. The greatest metmyoglobin formation was found at pHs of 5-6 (P<0.05). Metmyoglobin formation generally decreased as the pH value increased up to pH 11 (P<0.05). At acidic pHs, the proton-catalyzed displacement process was mainly responsible for promoting the autoxidation reaction of the oxymyoglobin (Kitahara *et al*., 1990). At alkaline pHs (9-11), the sharp decrease in metmyoglobin might be due to the enhanced formation of oxymyoglobin over the alkaline pH ranges (Eder, 1996). However, at extreme alkaline condition (pH 12), the formation of metmyoglobin increased (P<0.05). This was probably owing to the oxidation of oxymyoglobin to yield metmyoglobin promoted by the high oxygen tension (Kitahara *et al*., 1990). Under air-saturated conditions, oxymyoglobin oxidized easily to metmyoglobin with generation of superoxide anion (Kitahara *et al*., 1990). Low pH reduced the stability constant for heme-globin linkage and increased the autoxidation rate (Renerre *et al*., 1992). Livingston and Brown (1981); Govindarajan (1973) also reported that autoxidation of myoglobin became greater and faster as pH decreased. Susceptibility of myoglobin varied with species. Kitahara *et al*. (1990) found that bigeye tuna oxymyoglobin was much more prone to oxidation over the whole range of pH values (pH 5-12), compared with sperm whale oxymyogloin.

Tryptophan fluorescence intensity of sardine myoglobin at various pHs is depicted in Figure 26B. At pH 6-7, which was the physiological pH of sardine muscle, the heme-globin complex might be compacted and stabilized. The heme-globin associate/dissociate rate was generally low at neutral pH (Giddings, 1974). At acidic pH, the sharp increase in fluorescence intensity was found, especially with decreasing pH (P<0.05). 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. 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). An amphipathic □-helix has opposing polar and non-polar faces along its helical axis (Clayton and Sawyer, 1999). In an amphipathic peptide, tryptophan is moved progressively along the sequence from the non-polar face to the polar face of the peptide (Clayton and Sawyer, 1999). The fluorescence

spectroscopy of this amino acid offers a very powerful tool to monitor the changes in protein structure (Copeland, 1994). Fish myoglobins contained one or two tryptophan residues (Chanthai *et al*., 1996). Watts *et al*. (1980) reported that yellowfin tuna comprised one trytophan residue. From the result, increasing the proton concentration of an aqueous oxymyoglobin system at acidic pHs might influence globin conformation as well as apomyoglobin unfolding, resulting in the increase in fluorescence intensity. At basic pH (pH 8-12), the fluorescence intensity gradually decreased as pH increased ($P<0.05$). The conformational changes of protein in the way which hydrophobic interaction was buried inside the molecule could take place as shown by the lowered fluorescence intensity. Changes in conformation that accompany ligand binding and protein unfolding affected the environment of some or all tryptophan residue within the protein (Copeland, 1994).

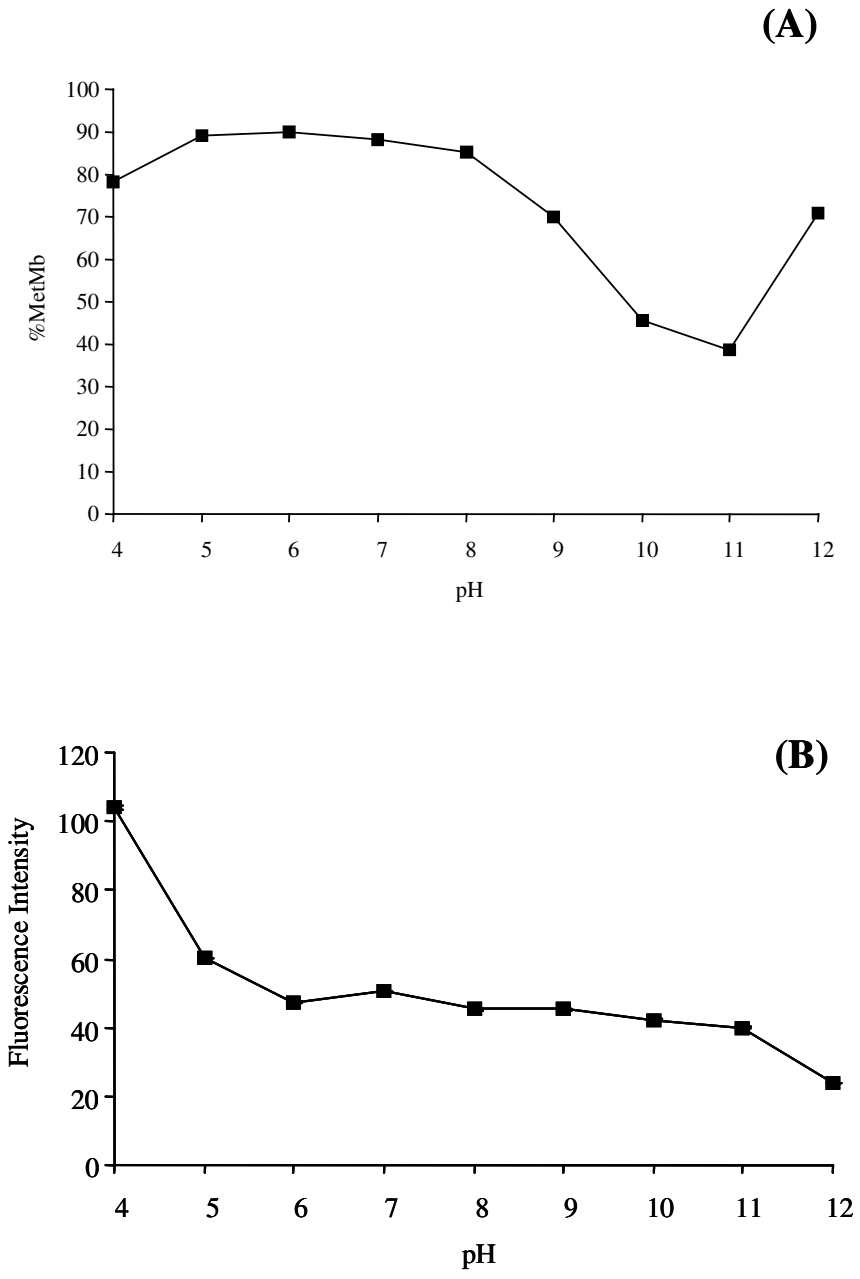


Figure 26. Effect of pH on the metmyoglobin formation (A) and tryptophan fluorescence intensity (B) of sardine myoglobin. Bars indicate the standard deviation from triplicate determinations.

Effects of pH on the absorption spectra of sardine myoglobin are shown in Figure 27. The soret peak of myoglobin was stabilized over the pH ranges of 6-9. It has been shown that apomyoglobin in solution with pH close to neutral has a compact and unique spatial

structure with an extended hydrophobic core (Chen *et al*., 2004). At very acidic or alkaline pHs, the soret peak tended to decrease. The disappearance of soret absorption band was found at extreme acidic pH (4) and basic pH (12). This indicated the destruction of heme protein. The α-helical portion of myoglobin might be weakened under these conditions. Myoglobin was able to unfold more easily, possibly by changing the α-helix of protein into a randomly coiled structure (Puett, 1973). The soret band of myoglobin results mainly from the interaction of the heme moiety with apomyoglobin, hence it can be used to monitor the unfolding of hemoproteins (Chen and Chow, 2001). Furthermore, Baron *et al*. (2002) also reported that heme protein degradation or the detachment of the porphyrin moiety from the globin was monitored by changes in the soret absorption band.

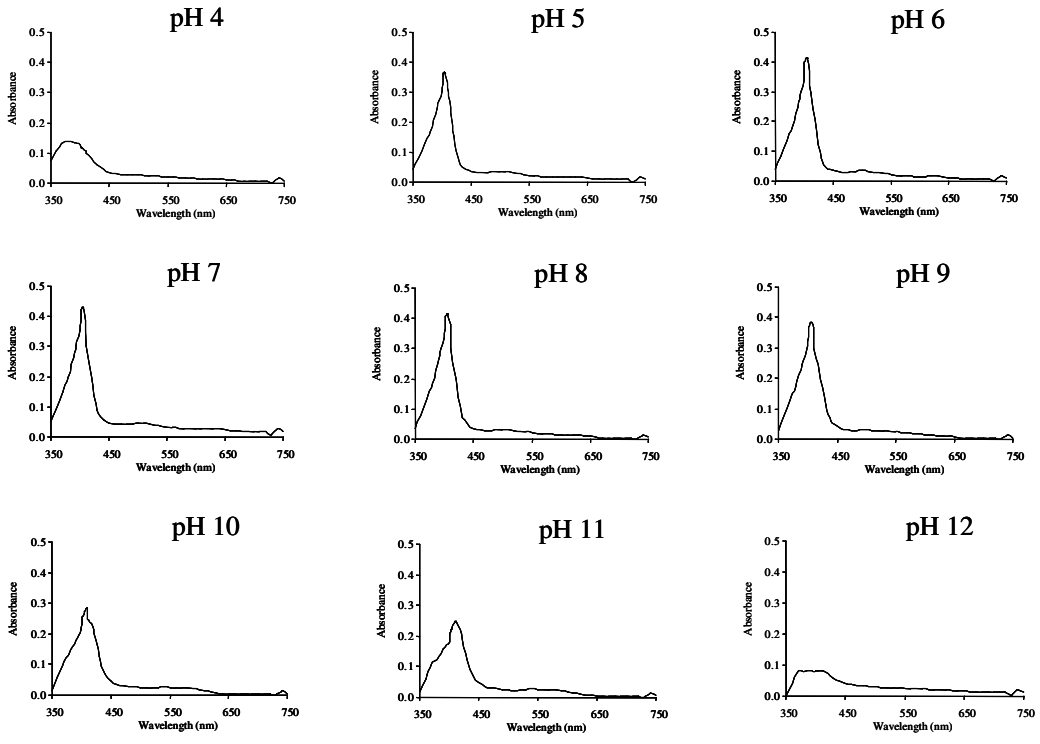


Figure 27. Effect of pH on the absorption spectra of sardine myoglobin.

Effect of temperature on myoglobin stability

Effect of temperature on the autoxidation of myoglobin is illustrated in Figure 28A. Metmyoglobin content increased obviously with increasing temperature and incubation time (P<0.05). No metmyoglobin formation was noticeable at temperature lower than 20°C. After incubation at 70°C for 10 min, the metmyoglobin increased by 9.81%, compared with that at 0° C. With the longer incubation time (30 min) at 70°C, the metmyoglobin content increased by 12.64%. Young and West (1996) reported that the higher the temperature, the higher the autoxidation rate of myoglobin was obtained. Furthermore, rising the temperature of fresh red meat tended to promote the autoxidation of myoglobin (Giddings, 1974).

The intrinsic tryptophan fluorescence intensity of sardine myoglobin was determined to monitor thermal unfolding or conformational changes of the tertiary structure. (Clayton and Sawyer, 1999; Van Dael *et al*., 1997). The fluorescence intensity of sardine myoglobin decreased with increasing temperature (P<0.05) (Figure 28B.). Additionally, the longer the incubation time, the greater the decrease in fluorescence intensity was observed. However, no marked change in fluorescence intensity of sardine myoglobin was found at temperature below 40°C for both incubation times (P>0.05). At higher temperature and longer time, the more loosely compact of globin might occur and the aggregation of globin might be enhanced via hydrophobic interaction of unfolded molecules. When heated, tryptophan and tyrosine residues normally shielded in the folded myoglobin molecule become exposed and subsequently underwent aggregation. Copeland (1994) reported that elevated temperatures, not only favor the unfolded state of a protein, but also greatly increase the rate of collisions between unfolded protein molecules. Chanthai *et al*. (1996) found that the fluorescence intensity of myoglobin from yellowfin tuna, bonito and sperm whale decreased linearly with increasing temperature from 10 to 63°C. The conformational changes of fish myoglobin was clarified to some extent by the partial thermal unfolding and refolding states (Chen *et al*., 2004). From the result, the lowest fluorescence intensity at 60-70°C was in agreement with the thermal denaturation temperature (60-76°C) (Figure 24).

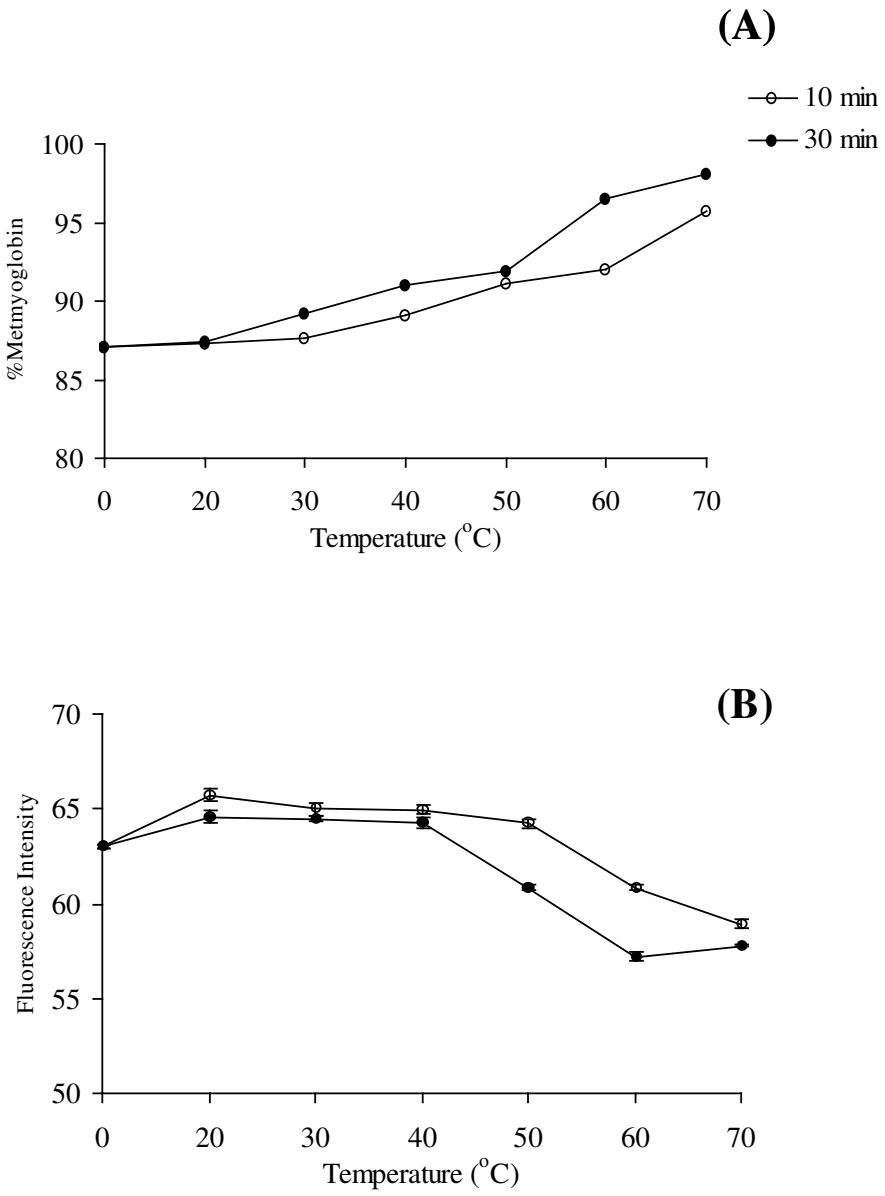


Figure 28. Effect of temperature on the metmyoglobin formation (A) and tryptophan fluorescence intensity (B) of sardine myoglobin. Bars indicate the standard deviation from triplicate determinations.

No changes in soret peak of myoglobin were observed over the temperature ranges of 0-60°C (data not shown). At 0-60°C, the longer incubation time had no effect on the peak height of soret band. Myoglobin might undergo a negligible conformational change at temperature below its denaturation temperature. Nevertheless, a slight decrease in soret peak height was found in the sample heated at 70°C for 30 min (data not shown). Ledward (1992a)

reported that the denaturation of myoglobin in meat tends to occur at temperatures of 60°C and above. Thus, high temperature might induce the conformational changes of myoglobin, which directly govern its properties.

6.5 Conclusion

Myoglobin isolated from the sardine dark muscle had the molecular weight of 15,300 daltons. Its absorption spectra depended on the form as well as oxidation states. Among all derivatives, metmyoglobin was more susceptible to denature as shown by the lowered transition temperature, compared to oxy and deoxy counterparts. Sardine myoglobin was prone to oxidation and denaturation at temperature above 40°C and very acidic or alkaline pHs.