# CHAPTER III
# EXPERIMENTAL

## 3.1 Chemicals and reagents

All chemicals and reagents used in this research were electrophoresis purity reagent grade and analytical reagent grade. Acrylamide, N,N'-methylene-bis-acrylamide, bromophenol blue, DEAE-cellulose, sodium dodecyl sulphate and Tris(hydroxymethyl)–aminomethane were obtained from Sigma Chemicals (USA). β-mercaptoethanol was derived from Acros Organic (USA). Coomassie brilliant blue R-250 was purchased from Fluka Chemika (Switzerland). Ampholytes, IEF-calibration kit and low molecular weight standard protein marker were obtained from Amercharm Bioscience (Sweden). The chemicals with analytical reagent grade including bovine serum albumin, horse heart myoglobin, ammonium sulfate, sodium dithionite and 3,5-dimethoxy-4-hydroxycinamic acid were obtained from Sigma Chemicals (USA). Acetic acid, glycerol, hydrochloric acid and methanol were purchased from Carlo Erba (Italy). DEAE-cellulose and Sephadex G-75® for column chromatography were products of Sigma-Aldrich (USA) and Amercharm Bioscience (Sweden), respectively.

## 3.2 Instruments

Instruments employed in this research consist of high speed refrigerated centrifuge, model Hitachi CR22GII (Hitachi Koki, Japan) and centrifuge model Centurion Scientific 1010 (Centurion Scientific, England) were used for remove all precipitate protein and used in ultrafiltration technique. The samples were separated and/or concentrated by using Amicon® Ultra-4, Millipore Corporation (USA) with molecular weight cut off 10 and 30 kDa. Deionized water was achieved by Simplicity Water Purification System, model Simplicity 185, Millipore Corporation (USA). Reverse osmosis water was achieved by RiOs™ 5, Millipore Corporation (USA). Dialysis tubing cellulose membrane, Sigma Chemical, (USA) was used for desalting of protein mixture solution. A freezer, model SF-C691 was used for keep all products. The myoglobin separation and its purity were determined by gel electrophoresis,

model Mini-Protein II Dual Slap and Power supply, model 1000/500, Bio-Rad Laboratories (USA). Glass columns, 100×1.75 cm i.d. and 50×1.5 cm i.d. (Pyrex, Thailand) were used for containing Sephadex G-75® and DEAE cellulose®, respectively.

The pH meter, Denver Instrument, Model UB-10 (USA) was used in all experiments. The spectral properties and its autoxidation rate of myoglobin were analysed using a Ultraviolet-Visible Spectrophotometer, Model 8453, AGILENT (Germany) and Spectrofluorophotometer, model RF-5301 Shimadzu, Shimadzu Corporation (Japan). Partial peptide sequence of myoglobin was determined by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) using Finnigan LTQ Linear Ion Trap Mass Spectrometer, Thermo Scientific (USA). Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) using an AXIMA Performance™ Mass Spectrometer, Shimadzu, Biotech (Japan) was used for molecular weight determination.

### 3.3 Fish muscle

Asian swamp eel (*Monopterus albus*) with an average body weight of 300-400 g were bought from a public market in Khon Kaen province, Thailand.

### 3.4 Extraction and isolation of fish myoglobin

The ordinary muscle from the swamp eel was removed free of gross fat, connective tissue, bone and skin. It was chopped coarsely and extracted with two volume of cold 10 mM Tris-HCl buffer (pH 6.8) by homogenization in a blender at low speed and the suspension was passed through folded cheesecloth. All steps above were done at 4°C. The insoluble material was removed by centrifugation at 10,000 ×g for 15 min at 4°C. The extracted solution containing the crude myoglobin represents at this step. This myoglobin solution was then subjected to further isolation step by fractionation with ammonium sulfate. The fractionation of myoglobin was subsequently carried out at 4°C, by adding 55 to 75% saturation of solid ammonium sulfate. The voluminous precipitate of the 55% salt saturated fraction, which probably contained much hemoglobin or other proteins, was discarded after centrifugation at 10,000 ×g for 20 min. Ammonium sulfate was then added to 75% saturation. The

precipitate protein obtained from salting out was collected after centrifugation at 10,000 ×g for 30 min. The pellet was dissolved in 20 mM Tris-HCl buffer (pH 6.8) and then dialyzed extensively at 4°C against 20 mM Tris-HCl buffer (pH 6.8); three times for 24 hours. The insoluble proteins which sometimes occurred in the dialysis bag while conducting the isolation step was removed by centrifugation. After determining the protein composition by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 15% gel) using horse heart myoglobin as references, the crude myoglobin solution can be concentrated by ultrafiltration technique using Amicon® with molecular weight cut-offs 10 kDa and stored at -30°C for purification. The stepwise for extraction and isolation of fish myoglobin is shown in Figure 3.1.

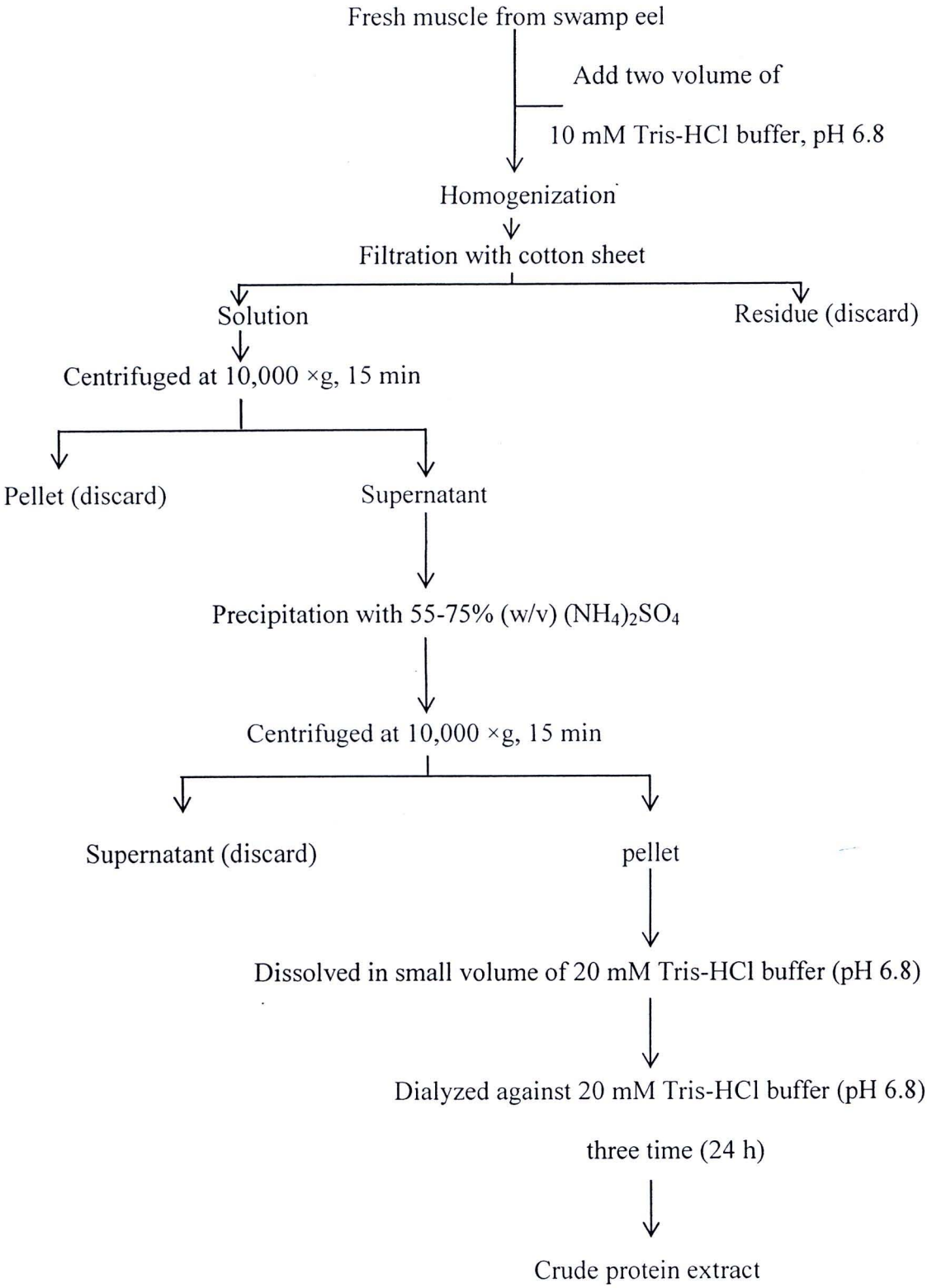


**Figure 3.1** Extraction and isolation procedure of myoglobin from swamp eel

### 3.5 Purification of fish myoglobin

After the extraction and isolation steps, the concentrated crude myoglobin was obtained. In this research, the purification of swamp eel myoglobin by several techniques including column chromatography and non-denatured polyacrylamide gel electrophoresis was performed as follow.

#### 3.5.1 Purification of fish myoglobin by column chromatography

Column chromatography was generally used for purification of myoglobin from sample matrices. In this research, the purification of fish myoglobin by column chromatography was carried out by two chromatographic steps.

**3.5.1.1 Purification of fish myoglobin by gel filtration chromatography followed by ion exchange chromatography.**

The purification of myoglobin was firstly achieved by gel filtration. The concentrated myoglobin was fractionated on a column (90 × 1.5 cm i.d.) of Sephadex G 75® and equilibrated with 20 mM Tris-HCl buffer (pH 6.8). About 0.5 mL of the myoglobin solution was applied onto the column and developed by the same Tris-HCl buffer (pH 6.8) at a flow rate of 0.33 mL/min. About 2 mL of eluate solution were collected. The myoglobin-containing fraction examined by the absorbance at 280 and 408 nm, and by SDS-PAGE, were pooled and subsequently concentrated by Amicon® with molecular weight cut-offs 10 kDa. The concentrated fractions obtained from Sephadex G 75® column were applied onto the weak anion exchange DEAE-cellulose column (50×1.0 cm i.d.) equilibrated with 20 mM Tris-HCl buffer (pH 6.8). The fractions of proteins were eluted by the same buffer at a flow rate of 0.33 mL/min. Each of 2 mL per fraction was collected.

**3.5.1.2 Purification of fish myoglobin by ion exchange chromatography followed by gel filtration chromatography.**

The fish myoglobin was purified using DEAE-cellulose column and then Sephadex G 75® column. About 0.5 mL of the crude myoglobin was introduced onto a 50 x 1.5 cm i.d. column of DEAE-cellulose equilibrated against 20 mM Tris-HCl pH 6.8. Each fraction was collected by stepwise elution (20 mM Tris-HCl buffer (pH 6.8)) at a flow rate of 0.33 mL/min. The myoglobin containing fractions were pooled and concentrated by Amicon® with molecular weight cut-offs 10 kDa. These

concentrated fractions were then chromatographed onto a 1.5 x 90 cm i.d. column of Sephadex G 75®. The procedures used for this purification step were carried out in the same manner as described above.

### 3.5.2 Purification of fish myoglobin by non-denatured polyacrylamide gel electrophoresis (Native PAGE)

#### 3.5.2.1 Optimization conditions of myoglobin separation on Native PAGE

Non-denatured polyacrylamide gel electrophoresis was developed for purification of swamp eel myoglobin by monitoring the appeared brown band on the unstained gel. In order to obtain appropriate conditions for their purification, the affecting parameters for electrophoresis separation including acrylamide concentration and applied separation voltage were investigated in this study.

**1) Acrylamide concentration**

Since separation resolution and efficiency of polyacrylamide gel electrophoresis rely on the gel pore size, the size of gel pore was investigated by variation of total gel concentration (%T). The protein mixture was applied on native PAGE with 7.5, 10 and 15% gel. The polyacrylamide gel preparation was described elsewhere. The applied voltage of 150 V was used for all gel concentration, observing the brown band of myoglobin that migrated through the unstained gel.

**2) Applied separation voltage**

An applied separation voltage could affect on separation efficiency and separation time. Hence, the applied separation voltage was performed with various voltages. This study was carried out with optimum gel concentration. The separation voltages of 150, 200 and 250 V were studied. Also, the separation time of the obvious brown band of myoglobin was investigated.

#### 3.5.2.2 Purification of fish myoglobin

The purification of fish myoglobin was carried out under the optimum conditions obtained from the optimization step. The concentrated crude myoglobin was loaded on an optimum gel concentration of acrylamide gel. The brown color band of crude myoglobin was migrated through the gel under the

optimum applied voltage until it reaches a position 1 cm. from the bottom of the gel. The brown band of myoglobin from unstained gel was excised from the gel and then extracted by grinding in 0.5 M Tris-HCl buffer pH 8.8. The extract solution was centrifuged at 10,000 ×g to remove gel residues. The concentrated brown band extract was then kept storing for identification and characterization.

### 3.6 Purity of fish myoglobin

From the purification step, purity of the purified myoglobin from column chromatography and non-denatured polyacrylamide gel electrophoresis were assayed for its purity by SDS-PAGE (4% stacking gel and 15% separating gel) compared with low molecular mass standard protein. The purity was also confirmed using absorption ratio at the Soret peak (407-415 nm) and the aromatic region (280 nm) by UV-Visible spectrophotometry.

### 3.7 Protein concentration determination

The total protein concentration was determined spectrophotometrically at 595 nm by Bradford assay using bovine serum albumin (BSA) as standard protein. The procedure was described as following.

#### 3.7.1 Preparation of standard and assay reagent

**1) Bradford reagent preparation**

100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 mL of 95% ethanol. Then 100 mL of 85% (w/v) phosphoric acid was added and adjusted to 1 liter with deionized water. The Bradford reagent was filtered through filter paper Whatman No.1 and kept in dark bottle at 4°C until use.

**2) Standard BSA preparation**

The stock standard solution of BSA (1000 mg/L) was prepared by dissolving 0.05 g BSA in 30 mL of 20 mM Tris-HCl buffer (pH6.8) and the volume was made up to 50 mL with the same buffer. The standard solution of BSA was kept at -20°C until use.

### 3.7.2 Assay procedure

The working standard solution was freshly prepared by adding the stock solution of 1000 mg/L BSA to 1.8 mL Bradford reagent and made up to 2 mL with 20 mM Tris-HCl buffer (pH 6.8) to provide standard solution concentrations of 0, 2, 4, 6, 8 and 10 μg/mL. After 10 min. incubation at room temperature, the absorbance of each BSA concentrations was measured at 595 nm. The calibration curve was created by plotting the change in absorbance at 595 nm versus protein concentration. To determine the protein concentration of sample, the sample was prepared in the same manner as described above and the obtained absorbance was interpolated from standard curve.

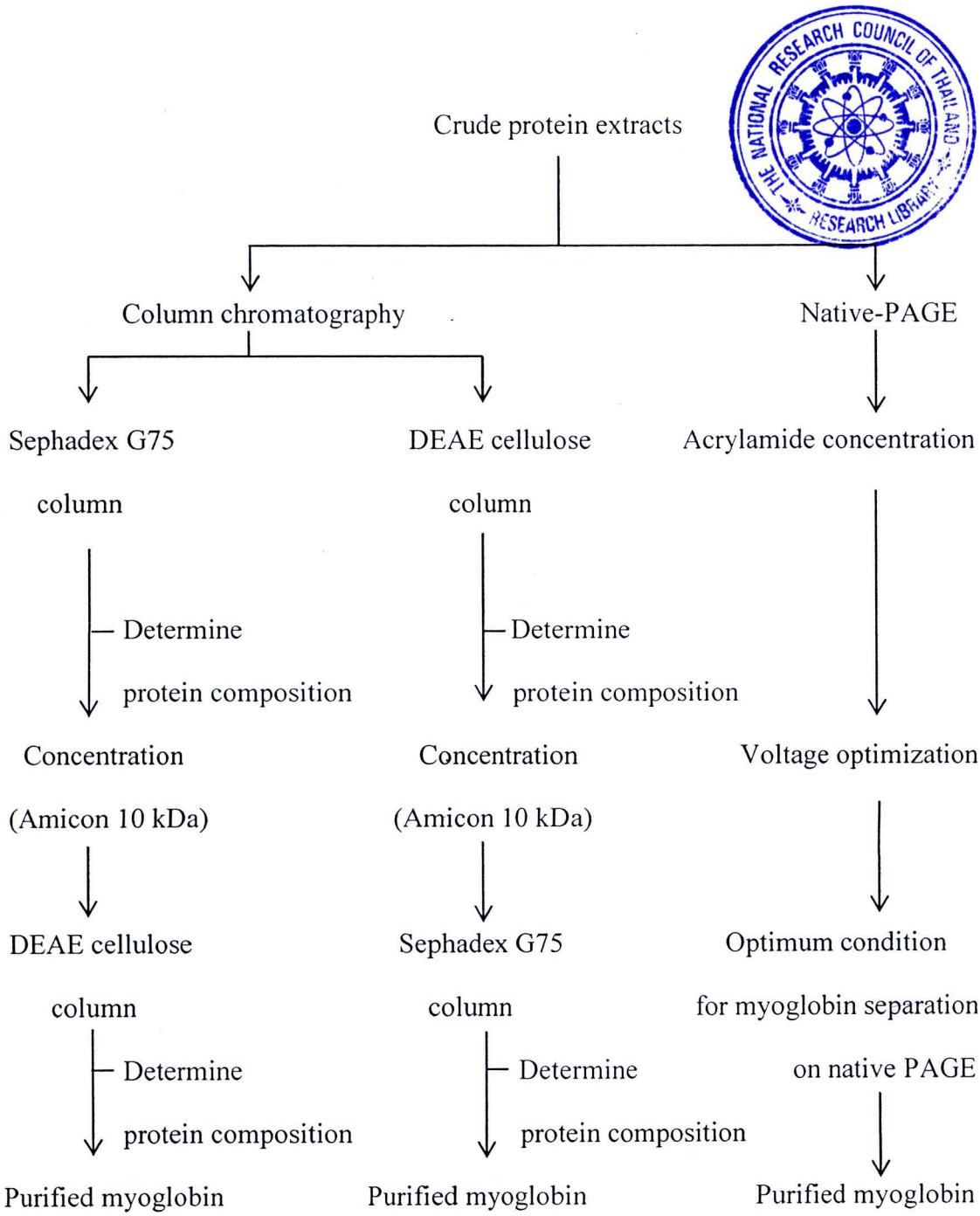




**Figure 3.2** The schematic methods for purification of myoglobin from swamp eel

### 3.8 Characterization and identification of fish myoglobin

The characterization and identification of fish myoglobin including molecular weight, isoelectric point, molecular absorption, tryptophan fluorescence quenching, autoxidation rate, extinction coefficient and partial peptide sequencing by LC-MS/MS were investigated.

#### 3.8.1 Molecular weight determination

The fish myoglobin was estimated their molecular weight by using mobility calculation ($R_f$). The purified myoglobin and low molecular weight protein markers were separated on SDS-PAGE (15% gel) which performed at 150 V. The $R_f$ value of the purified myoglobin was interpolated from the standard curve of log molecular weight of the set of low molecular mass standard and their $R_f$ values. A set of the low molecular weight markers (kDa): phosphorylase B (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), soybean trypsin inhibitor (20), α-lactalbumin (14.4) was used for making the standard curve.

Furthermore, in order to obtain an accurate molecular mass, the purified fish Mb was also measured by Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) using an AXIMA Performance$^{TM}$ mass spectrometer (Shimadzu biotech, Japan) with 3,5-dimethoxy-4-hydroxycinamic acid (Sigma-Aldrich) as a matrix. Spectrum was recorded in linear mode with an acceleration voltage of 377 mV. To prepare sample for mass spectrometry, 1 μL of the matrix solution was spotted on the MALDI plate. The 1 μL of the purified Mb solution was then spot quickly onto the drop of the matrix solution and then air dried at room temperature to allow sample crystallization prior to insertion into the instrument.

#### 3.8.2 Isoelectric point determination

Determination of an isoelectric point (pI) value of the fish myoglobin was performed by isoelectric focusing method (IEF). The horizontal slab gel IEF (5.5% acrylamide) with pH gradient from 3.5-10 ampholytes was used to determine pI value of fish myoglobin. About 7μL of the purified myoglobin was applied to the gel and higher voltage was applied (100 V for 25 min, 200 V for 25 min and 800 V for 30 min). Isoelectric point of the purified myoglobin was estimated from linear

extrapolation of the calibration curve relating electrophoretic mobility ($R_f$) from a cathode to an anode to pI of reference proteins (IEF calibration kit protein marker); amyloglucosidase (3.5), glucose oxidase (4.2), trypsin inhibitor (4.5), β-lactoglobulin (5.15, 5.3), carboanhydrase (6.0), horse myoglobin (6.8, 7.35), lentil lectin (7.75, 8.0, 8.3), ribonuclease A (9.45), cytochrome C (10.65).

### 3.8.3 Molecular absorption

The molecular absorption measurements of the crude myoglobin, purified myoglobin (20 ppm), horse heart myoglobin (20 ppm) and bovine serum albumin solution (20 ppm) were carried out using an UV-Visible spectrophotometer. About 2.5 mL of the protein solutions was transferred into a quartz cell (light path, 1.0 cm). Absorption spectra of the protein solutions were recorded from 250 to 700 nm with 20 mM Tris-HCl buffer (pH 6.8) as a blank.

### 3.8.4 Tryptophan fluorescence

Fluorescence measurement was performed with a spectrofluorophotometer. About 2.5 mL of the purified myoglobin (20 ppm), horse heart myoglobin (20 ppm) and bovine serum albumin (20 ppm) was approximately used in a 1 cm path length cell. Fluorescence excitation was set at 280 nm for tryptophan fluorescence intensity. Both the relative fluorescence intensity and maximum emission wavelength of the proteins were recorded.

### 3.8.5 Partial peptide sequence analysis

Partial peptide sequence of the purified myoglobin was carried out by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). This purified myoglobin was separated on SDS-PAGE (15% gel). With Coomassie blue staining, the single protein band was obtained. The protein band was excised from the gel and collected in each microtube containing deionized water. The excised protein band was determined the peptide fragment using in-gel digestion with trypsin and analyzed the peptide fragments by LC-MS/MS. Based on the LC-MS/MS results, a search in nr.FASTA by Biowork$^{TM}$ 3.1 SR1 (ThermoFinnigan) was performed to identify the

protein bands. The LC-MS/MS and database search were done at the Genome Institute, BIOTEC, Pathumthani, Thailand.

### 3.8.6 Determination of molar extinction coefficient

The myoglobin used in this study was isolated by column chromatography from the swamp eel. The stock solution of fish myoglobin was prepared by dissolving 1 mg of myoglobin in 5 mL of 20 mM Tris-HCl buffer (pH 6.8). The protein solution was filtered by Whatman No. 42 filter paper and the final volume was made up to 10 mL by adding the same buffer. The protein content of the solution was determined by Bradford protein assay. The protein solution was diluted to 10, 20, 30, 40 and 50 mg/L. All of the myoglobin solutions were monitored UV-Visible absorption at 280 and 408 nm using a spectrophotometer using 20 mM Tris-HCl buffer (pH 6.8) as a blank. The molar extinction coefficient for each wavelength was calculated from slope of the straight curve by plotting the changes in absorbance unit versus its protein concentration.

### 3.8.7 Determination of autoxidation rate constant

#### 3.8.7.1 Preparation of myoglobin solution

The stock solutions of fish myoglobin and horse heart myoglobin were prepared by dissolving 20 mg of each myoglobin in 20 mL 0.2 M phosphate buffer (pH 6.8). Both protein solutions were filtered by Whatman No. 42 filter paper and made up final volume to 20 mL by the same buffer. The protein contents of these stock solutions were determined using Bradford protein assay. The protein sample was adjusted to be 0.6 mg/mL by adding 0.2 M phosphate buffer (pH 6.8) and kept at -20$^0$C prior to analysis.

#### 3.8.7.1 Preparation of oxymyoglobin

The stock protein solutions (0.6 mg/mL) were used to prepare their oxymyoglobin. An aliquot (3 mL) of the protein was converted to its oxy-form by the addition of 50 μL of fresh sodium dithionite solution (0.1 mg/mL) and gently agitated with urging of oxygen gas (about 2-3 min) until the bright red colored was appeared.

### 3.8.7.2 Autoxidation rate measurement

The autoxidation rate of myoglobin was measured in the presence of sodium dithionite in 0.2 M phosphate buffer pH 6.8 at 37°C. About 3 mL of oxymyoglobin was quickly transferred to a quartz cell and place in thermostat connecting with UV-visible spectrophotometer. The changes in the absorption spectrum region from 450 to 650 nm were recorded at interval of 5 min for 2 h, using 0.2 M phosphate buffer pH 6.8 as a blank. The sample was subjected to the absorbance measurement at 581 nm (α peak). The absorbance data were analyzed by plotting in terms of ($MbO_{2t}$ / $MbO_{2o}$) vs. time where $MbO_{2t}$ represents the concentrations of oxymyoglobin at any time ($t$), and $MbO_{2o}$ was the initial concentration of oxymyoglobin. The slope of the straight curve was taken as the apparent first-order rate constants ($k_{obs}$). The ratio was monitored by measurement at 581 nm for the initial time ($A_o$), at any time during the kinetic run ($A_t$) and was expressed as $\ln\{(A_t)/(A_o)\}$.