CHAPTER V
CONCLUSION

5.1 Extraction, isolation and purification of swamp eel myoglobin

Myoglobin from Asian swamp eel (*Monopterus albus*) could be extracted by homogenization with 20 mM Tris-HCl buffer (pH 6.8). The extracted solution was precipitated with 75% (w/v) ammonium sulfate. The precipitated protein was obtained from salting out and dissolved in a minimal volume of 20 mM Tris-HCl buffer (pH 6.8). The protein solution exhibited the absorption spectrum maxima at 280, 413, 539 and 581 nm. It is shown that oxy-form of myoglobin was present in the protein solution. This protein solution (crude protein) appeared as red brown color was used for myoglobin purification and characterization.

In these studies, the column chromatography and non-denatured polyacrylamide gel electrophoresis were used for purification of myoglobin from the swamp eel. The crude myoglobin was separated on DEAE cellulose column (weak anion exchange) and Sephadex G-75® column (gel filtration), respectively. During purification, the fractions were obtained from the column and measured at 280 and 408 nm using a UV-Visible spectrophotometer. The fractions with high absorbance at 408 nm obtained from DEAE cellulose column were also subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The SDS–PAGE pattern of the fractions was presented only one band of protein with molecular weight around 15.4 kDa. Alternatively, non- denatured poly acrylamide gel electrophoresis (native-PAGE) was developed to purify myoglobin from the crude protein. This result demonstrated that 15% acrylamide concentration and 250 V separation voltages were suitable for purify myoglobin. After separation on the optimum condition, the brown color band was excise from native gel and extracted by grinding in 0.5 M Tris-HCl buffer pH 8.8. The extracted protein obtained from native gel gave only one band with molecular weigh (MW) of about 15.4 kDa, which corresponded with purified myoglobin from column chromatography. From the purification steps, it was concluded that the both column chromatography and native PAGE method could be

used to separate the crude protein extract to get the purified myoglobin. The native PAGE method was a simple and method for purification of myoglobin and the yield of the procedure was higher than column chromatographic method.

5.2 Characterizations of swamp eel myoglobin

The purified myoglobin obtained from both column chromatographic method and native-PAGE method was used for characterize the physical and chemical properties. The physical properties of purified myoglobin were characterized in terms of their molecular weight, tryptophan fluorescence, extinction coefficient, partial peptide sequences and the chemical property was studied in term of the autoxidation rate constant.

Firstly, molecular weight of the purified myoglobin was determined by using SDS-PAGE (15% gel) method and MALDI-TOF-MS method which was found to be 15.43 and 15.52 kDa, respectively. The pI values of this fish myoglobin were achieved from isoelectric focusing technique appeared as two isoform around 6.40 and 7.12. The spectral properties of fish myoglobin were characterized by using UV-Visible spectrometry and spectrofluorophotometry. The UV-Visible absorption spectrum of the fish myoglobin exhibited maximum absorption at 280 and 408 nm, for protein absorption and the Soret peak of myoglobin, respectively. The fluorescence spectrum of the purified myoglobin at 325 nm showed lower intensity than that of BSA, it was corresponding to the fluorescence spectrum of horse heart myoglobin. The fluorescence intensity of myoglobin was quenched by heme group. Therefore, it is proposed that the fish myoglobin has the heme pocket. The fluorescence intensity of purified myoglobin obtained from native PAGE at 325 nm was lower than that of myoglobin obtained from column chromatography. So, myoglobin obtained from native page was found to be the unfolded protein by heating joules effect of electrophoresis.

Molar extinction coefficients (ε) of the fish myoglobin could be calculated from the slope of the straight curve (myoglobin concentration (M) vs absorbance unit (AU)). The extinction coefficients were found to be 2.22×10^4 $M^{-1}cm^{-1}$ at 280 nm and 4.80×10^4 $M^{-1}cm^{-1}$ at 408 nm. These coefficients were used to determine myoglobin concentration by using Lambert-Beer law from the equation of $A = \varepsilon bc$.

The autoxidation rate of myoglobin reflects to changing rate of oxymyoglobin to metmyoglobin, and it was investigated by using UV-Visible spectrometer at 581nm. The rate constant (k_{obs}) is calculated from the change of spectrum using first order plot (Time (h) vs $(MbO_2)_t/(MbO_2)_0$). The k_{obs} values of the autoxidation rate of the fish myoglobin and horse heart myoglobin at 37 °C in 0.2 M phosphate buffer pH 6.8 were found to be 1.42 h^{-1} and 1.08 h^{-1}, respectively. The autoxidation rate of the swamp eel myoglobin was higher than that of horse heart myoglobin. Therefore, this fish myoglobin was lowed oxygen affinity than horse heart myoglobin. The amino acid composition of fish myoglobin was determined by trypsin digestion and LC-MS/MS analysis which performed at the Genome Institute, BIOTEC, Pathumthani, Thailand. The sequence tags from LC-MS/MS are found six partial peptide fragments. Each of peptide fragments was identified by using the Turbo SEQUEST Algorithm in the BioWorks™ 3.1SR1 sofware package (Thermo Electron) and nr.fasta database. The fragments were identical to myoglobin from marine fish species; sea raven *(Hemitripterus americanus)*, yellowfin tuna (*Thunnus albacores*) and blue marlin (*Makaira nigricans*). Therefore, it concludes that the purified protein is the fish myoglobin and supposes that some structural and functional properties of swamp eel myoglobin are similar to those of marine fish myoglobins.