CHAPTER I
INTRODUCTION

1.1 Rationale and Problem

Myoglobin is a small globular protein present in a wide variety of species involving in an oxygen supply near the oxidative capacity of muscle and deliveries oxygen by facilitated diffusion (Conley & Jones, 1996). The richest sources of myoglobin are the aerobic muscle and heart ventricle of mammals that undertake extended breath-hold dives such as whales, seals and porpoises (Alexander, 1975). Also, this protein is found in reptiles, amphibians, teleosts, chondrichthid fish and lower animal species (Madden, Babcock, Vayda, & Cashon, 2004). Structurally, the X-ray crystallographic analysis indicates that myoglobin is a relatively, compact and globular protein which consists of a single polypeptide chain and the heme as a prosthetic group (Vijayan & Salunke, 1984; Hayashi & Hisaeda, 2002; Kong & Yu, 2007). The heme group composes of protoporphyrin organic part and a central iron atom (Paol, Marles-Wright, & Smith, 2002). The iron atom in the heme is coordinated to the four nitrogen atoms of a porphyrin and also to a nitrogen atom of a histidine amino acid residue in the myoglobin. The sixth coordination position around the iron is occupied by O_2 when the myoglobin is oxygenated (Zhieng, Liu, & Cai, 2005). The myoglobin functions depend critically on the chemical equilibrium of oxygen binding to the iron that is chelated to the heme group within the protein (Oleka et al, 2005). Therefore, myoglobin plays an important role in muscle cells by both temporary storing and transporting dioxygen. Likewise, myoglobin has been considered to be a free radical scavenger for protecting cell damage (Yang & Bono, 1993). Since some myoglobin properties are affected by the physiological ecology of animals, different kinds of animals living in different habitat show a difference in structural and functional properties of myoglobins (K.Nienhaus, Don, Deng, & G.Nienhaus, 2002; Madden et al., 2004; Oleka et al., 2005). The myoglobin content in different animal also significantly correlates with O_2 diffusion and their O_2 demand. In addition, 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 (Gidding, 1973; Livingston & Brown, 1981; Tamburrini, Romano, Giardina, & Prisco, 1999).

As well known, all functions of myoglobin are related with their structures. The primary structure (Suman, Joseph, Steinke, & Fontaine, 1981), three-dimensional structure (Kendrew et al., 1960), structural stability (Bellezza et al., 2007), spectral properties (K.Nienhaus & G.Nienhaus, 2005), oxygen binding properties (Brunori, 1995) and autooxidation mechanism (Brantley, Smerdon, Wilkinson, Singleton, & Olson, 1993) of myoglobin from various animals have been studied.

For both functional and structural studies, isolation and purification of myoglobin are important steps. These processes usually involve steps in which extraction with buffer, salt fractionation, and chromatography such as gel filtration, ion exchange and FPLC (Brown, 1996; Wan, Twitchett, Eltis Mauk, & Smith, 1998; Hayashi et al., 2002). All of these methods take a long time for purification besides high loss of protein mass along the entire procedure. However, some peculiar structural characteristic or physical–chemical property allows a special technique for purification (Loun, Copeland, & Sedor, 1996; Yang & Lin, 2002).

Recently, several techniques have been developed for separation and purification of myoglobin from any matrix in order to improve analysis time and increase protein concentration with high purity. These techniques were ultrafiltration (Loun et al., 1996), affinity chromatography (Qiu et al., 1998), two-dimensional gel electro phoresis (Rill & Al-Sayah, 2004) counteracting chromatographic electrophoresis (Chidambara-Raj & Hunter, 1992) counter current chromatography (Ching-Wei & Tiing, 2007) and centrifugal partition chromatography (Sutherland et al., 2008). In addition, SDS-PAGE (Godecke et al., 1999), spectrophotometry (K.Nienhaus & G.Nienhaus, 2005) and LC-MS/MS (Sutherland et al., 2008; Johan, Wouter, Klaus, & Gerhardus, 2006, Johan, Wouter, Klaus, & Gerhardus, 2006) were also used for myoglobin identification and characterization.

The aim of this research was to investigate structural and functional properties of in-land fish myoglobin. Since they have diverse range of habitats, fish species provide a good system in which to study the interrelationship between structure and function of myoglobin. We, therefore, are interested in the properties of myoglobin

from Asian swamp eel (*Monopterus albus*). This kind of fish can breathe air, allowing them to survive in deoxygenated water for long periods (Siang, Yee, & Seng, 2007). Since, the functional role of myoglobin has been considered to be cellular oxygen storage and facilitated O_2 diffusion. The swamp eel myoglobin may have some interesting characteristic and their physico-chemical properties. However, to our knowledge, there have not been previously reported yet the study of structural and functional properties of myoglobin from swamp eel.

Herein, myoglobin of swamp eel was isolated and purified by fractional ammonium sulfate precipitation and two-step chromatographic procedure. In addition, the purification of myoglobin by non-denatured polyacrylamide gel electrophoresis was also undertaken for comparison. This was a new separation and purification method developed in this research work. The structural properties of the myoglobin were characterized using UV-Visible spectrophotometry, spectrofluorometry, isoelectric focusing and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Functional property of the myoglobin was also an important point of view to be investigated.

1.2 Objectives

1.2.1 To investigate extraction, isolation and purification processes of myoglobin from Asian swamp eel by column chromatography and non-denatured polyacrylamide gel electrophoresis.

1.2.2 To obtain the optimal conditions of column chromatography and non-denatured polyacrylamide gel electrophoresis for their purification methods.

1.2.3 To study the separation patterns of myoglobin from the swamp eel on column chromatography and non-denatured polyacrylamide gel electrophoresis.

1.2.4 To investigate spectral properties of the swamp eel myoglobin by spectrophotometry and spectrofluorometry.

1.2.5 To determine molecular weight of the swamp eel myoglobin by using standard curve method and mass spectrometric data.

1.2.6 To determine pI value of the swamp eel myoglobin using isoelectric focusing method.

1.2.7 To determine partial peptide sequences of the swamp eel myoglobin by LC-MS/MS.

1.2.8 To determine autoxidation rate constant of the swamp eel myoglobin using an UV- Visible spectrophotometer.

1.3 Limitations of the study

1.3.1 Extraction, isolation and purification of myoglobin from Asian swamp eel (*Monopterus albus*).

1.3.1.1 Study on extraction and isolation of myoglobin from the swamp eel using cold buffer extraction and precipitation of a crude protein with ammonium sulfate.

(1) Protein composition

(2) Protein concentration

1.3.1.2 Study on purification of the swamp eel myoglobin using column chromatography and non-denatured polyacrylamidegel electrophoresis.

(1) Separation pattern of myoglobin

(2) Purity of the purified myoglobin

1.3.2 Characterizations of myoglobin from the swamp eel.

1.3.2.1 Study on physical properties of the swamp eel myoglobin.

(1) UV-Visible absorption spectrum

(2) Tryptophan fluorescence

(3) Molecular weight

(4) pI value

(5) Partial amino acid sequence

(6) Molar extinction coefficient

1.3.2.2 Study on chemical properties of the swamp eel myoglobin.

(1) Autoxidation rate constant

1.4 The anticipated outcomes

1.4.1 Isolation and purification of myoglobin can be obtained.

1.4.2 Analysis of myoglobin properties can be obtained.

1.4.3 Chromatographic and electrophoresis techniques for myoglobin analysis can be a choice of use.

1.4.4 Identification of partial amino acid sequence of the swamp eel myoglobin can be done.