CHAPTER IV
RESULTS AND DISCUSSION

4.1Extraction and isolation of myoglobin from swamp eel

Extraction and isolation of Asian swamp eel (*Monopterus allbus*) myoglobin was an important method to separate myoglobin from undesirable proteins and to concentrate it prior to purification steps. Fish myoglobin was extracted from red muscle of swamp eel by homogenization with cool buffer (20 mM Tris-HCl buffer, pH 6.8). The most of myoglobin was co-precipitated with other proteins in the range of 55-75% (w/v) saturated ammonium sulfate. This concentrate crude protein was used for myoglobin purification. The precipitated protein appeared as a red-brown color. The protein composition of the crude myoglobin was determined by using SDS-PAGE. There were many protein bands with molecular weight (MW) of $\leq$14.4 kDa to $\geq$94 kDa as shown in Figure 4.1a. The UV-Visible absorption spectrum of crude myoglobin which exhibited by tryptophan and tyrosine peaked at 280 nm and the Soret band of this crude myoglobin maximum absorption at 413 nm. In addition, the spectrum pattern of the crude myoglobin was also found corresponding to oxymyoglobin. The charge transfer peaks were found at 581 nm (α peak) and 539 nm (β peak) as shown in Figure 4.1b.

4.2 Purification of myoglobin from swamp eel

After obtained the crude myoglobin from extraction and isolation steps(55-75% (w/v) ammonium sulfate), the myoglobin purification was performed by using various techniques. Two procedures were used to purify myoglobin normally, column chromatography and non-denatured polyacrylamide gel electrophoresis. In all cases, the goal is to eventually separate myoglobin from other proteins.

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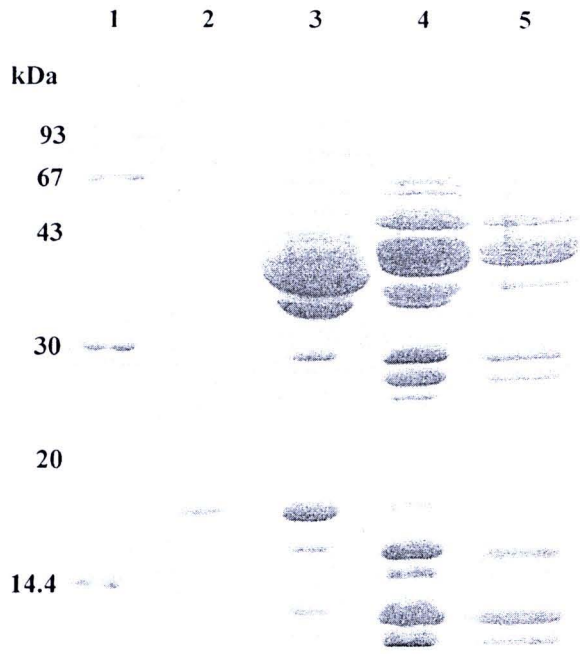

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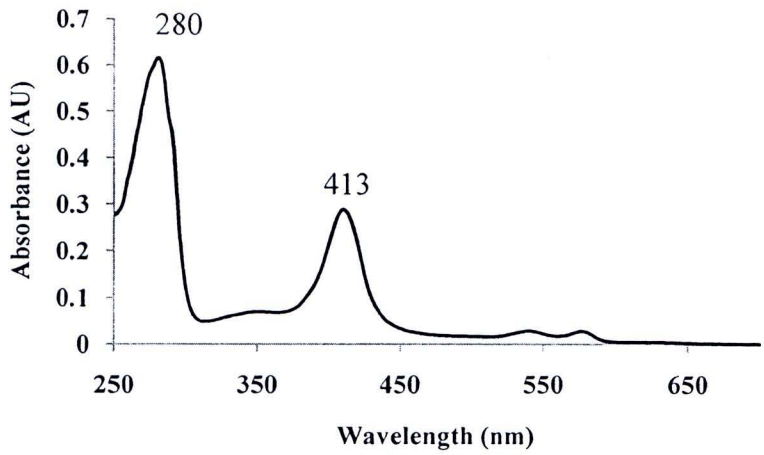


Figure 4.1 SDS-PAGE of supernatant (lane 4, 6) and pellet (lane 3, 5) obtained from swamp eel myoglobin after centrifugation and precipitation with 0-55% (lane 3,4) and 55-75% $(NH_4)_2SO_4$ (lane 5, 6) compared to protein molecular mass standards (lane 1), horse heart myoglobin (lane 2). (b) UV-Visible spectrum of the solution of the precipitate obtained from 55-75% salt saturation dissolved in 20 mM Tris-HCl buffer (pH 6.8).

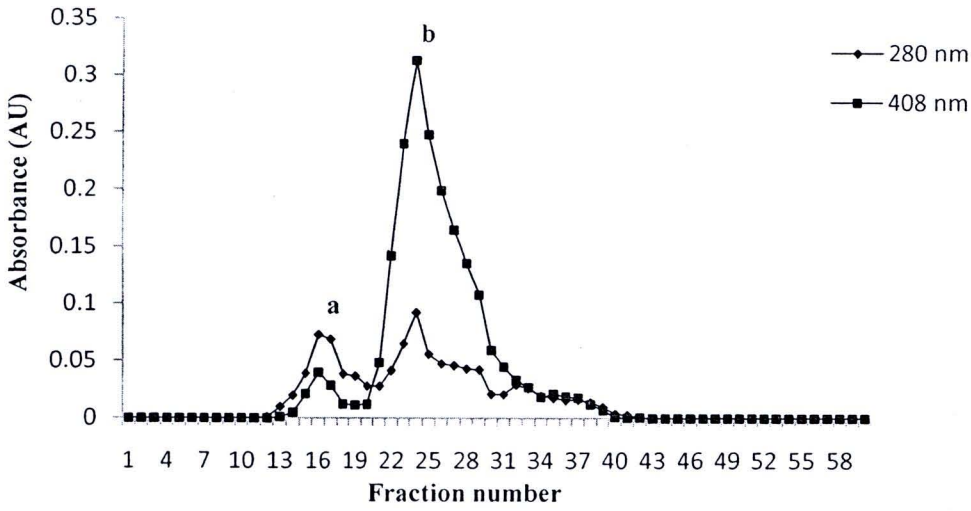
4.2.1 Purification of myoglobin by column chromatography

4.2.1.1 Purification of myoglobin by Sephadex G-75 and followed by DEAE-cellulose

The crude myoglobin (55-75% (w/v) saturated ammonium sulfate) was separated on Sephadex G-75 column by eluting with 20 mM Tris-HCl (pH 6.8). The elution profiles of crude myoglobin from this column showed two major peaks (peak a and b) exhibiting UV-Visible absorption at 280 nm and 408 nm (Figure 4.2 a). The fraction number 16 from peak a, fraction number 22, 25 and 28 from peak b were used to determine protein compositions using SDS-PAGE. The fraction 16 probably belonged to high molecular weight proteins. This fraction contained many bands of protein impurities. Peak b probably contained myoglobin judging from its color and its Soret absorptivity at 408 nm was higher than that of first peak. However, they still contained many protein impurities as demonstrated in Figure 4.2 b. The fractions (fraction 22-40) from peak b were concentrated using Amicon® with molecular weight cut off 10 kDa.

Furthermore, the concentrated fractions were subsequently introduced into the DEAE cellulose column. The elution profile from this column showed a single peak of chromatogram (Figure 4.3 a). The fraction number 11, 13 and 16 were analyzed on SDS-PAGE (Figure 4.3 b). These fractions contained six protein bands which had the molecular weight higher than 14.4 kDa. The Soret absorption at 408 nm was found from the solution in fraction number 13, indicating a higher concentration of myoglobin. From SDS-PAGE, the major protein band of fraction number 13 was found at about 15.4 kDa. The molecular mass of this band was similar to fish myoglobin. From the results, this procedure could not be successfully used for purification of myoglobin from swamp eel because the solution in fraction number 11, 13 and 15 from the peak it contained six protein bands.

(a)



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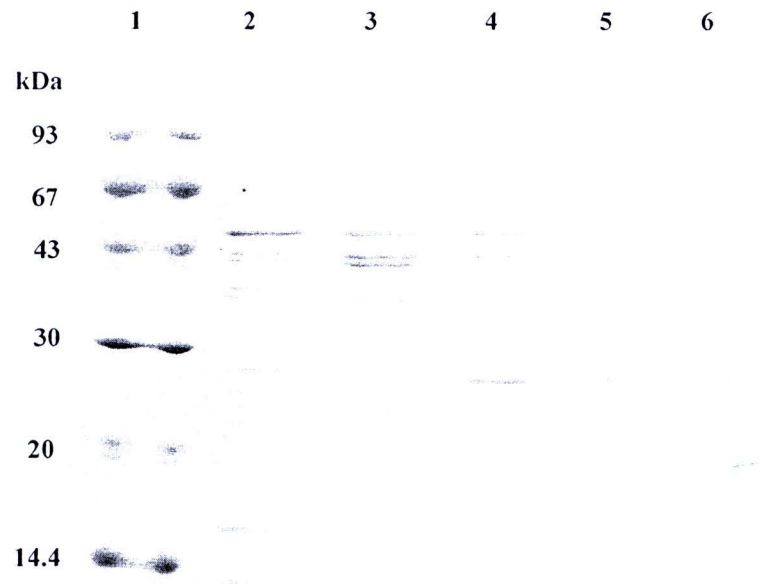
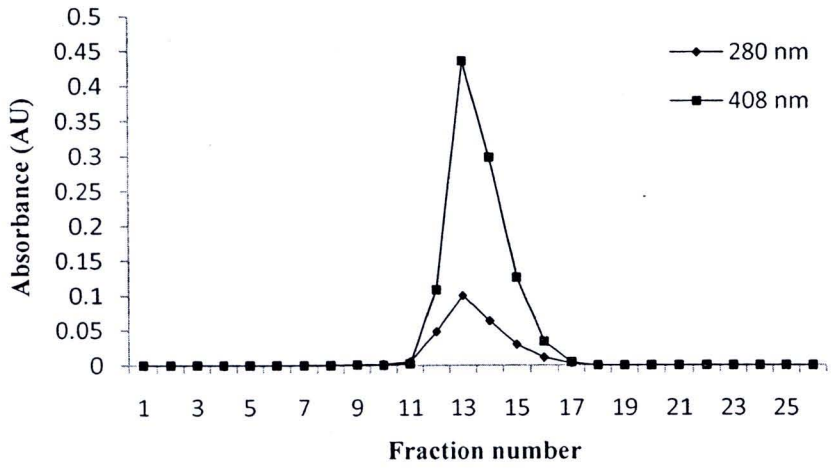


Figure 4.2 (a) Elution profile of the crude myoglobin obtained from Sephadex G-75 column chromatography. The eluate was measured at 280 nm and 408 nm. (b) SDS-PAGE of fractions that obtained from Sephadex G-75 column; lane 2: the crude extract of myoglobin, lane 3: fraction no. 16, lane 3, 4, 5: fraction no. 22, 25 and 28, respectively, compared to low molecular mass standard protein (lane 1).

(a)



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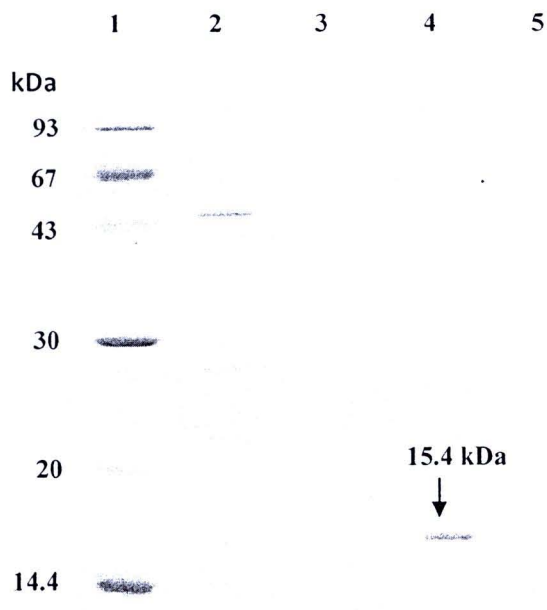


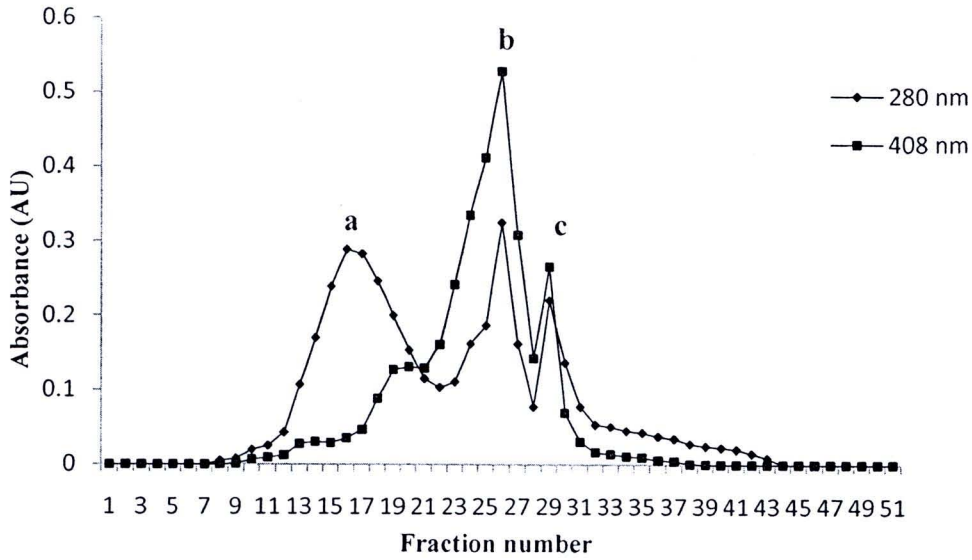
Figure 4.3 (a) Elution profile of the main fraction containing myoglobin obtained from DEAE cellulose column chromatography. The eluate was measured at 280 nm and 408 nm. (b) SDS-PAGE of fractions that obtained from DEAE cellulose column; lane 2: the crude extract of myoglobin, lane 3, 4, 5: fraction no. 11, 13 and 15, respectively, compared to protein low molecular mass standard (lane 1).

4.2.1.2 Purification of myoglobin by DEAE-cellulose and followed by Sephadex G-75

In section 4.2.1.1, that method was not suitable for purification of myoglobin from swamp eel. The purification of myoglobin was again developed from crude myoglobin precipitated with 55-75% (w/v) ammonium sulfate using DEAE-cellulose column and followed by Sephadex G-75 column. Firstly, the crude myoglobin was separated by DEAE-cellulose column, three main peaks were seen by UV-Visible absorption at 280 nm and 408 nm (Figure 4.4 a). The fraction number 23-28 and 28-43 of the peak b and peak c, respectively were judged to be the main part of myoglobin because of their high UV-visible absorption at 408 nm. The protein composition was also determined by a SDS-PAGE (Figure 4.4 b), the protein bands from fraction13-29 were monitored. Peak a was found to contain many protein bands having molecular weight less than 93 kDa. Peak b and peak c were found to contain five protein bands and the major band was found at 15.4 kDa. The molecular mass of the major band was similar to myoglobin from fish species.

The main myoglobin fractions (peak b and c, fraction number 23-40) were subsequently introduced onto a Sephadex G-75 column. After separation, the fraction number 26-33 of elution profiles (Figure 4.5a) that gave strong absorption at 408 nm was analyzed on SDS-PAGE. It contained single protein band with molecular weight about 15.4 kDa (Figure 4.5b). Therefore, column chromatographic method was appropriate to purify myoglobin from the swamp eel. Characterizations of this protein were then performed by using various techniques. The characterization parameters including molecular mass, isoelectric point, spectral properties, extinction coefficient and autoxidation rate constant of the purified myoglobin are investigated.

(a)



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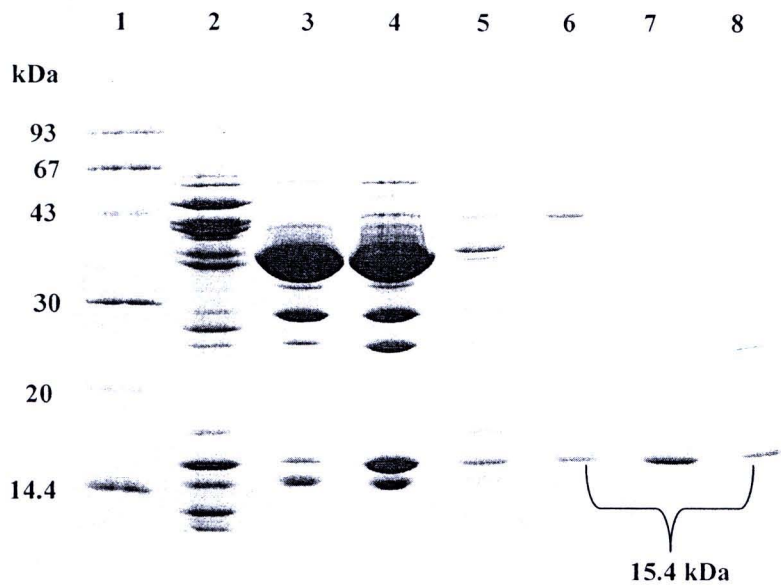
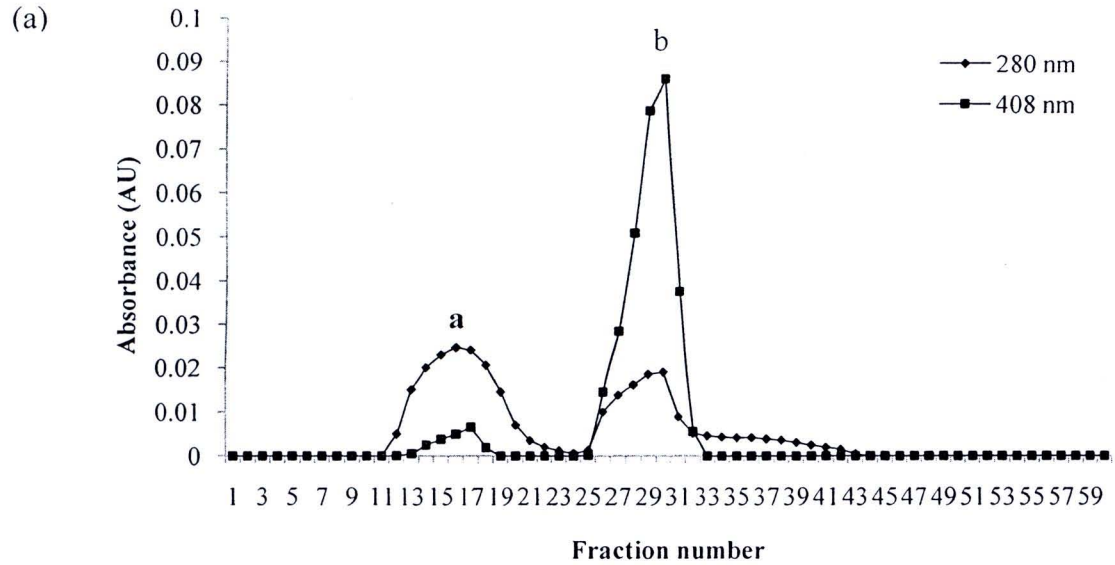


Figure 4.4 (a) Elution profile of crude myoglobin obtained from DEAE-cellulose column chromatography. The eluate was measured at 280 nm and 408 nm. (b) SDS-PAGE of fractions obtained from DEAE cellulose column; lane 2: the crude extract of myoglobin, lane 3, 4: fraction no. 13 and 15, lane 5, 6, 7, 8: fraction no. 22, 23, 26 and 28, respectively, compared to protein low molecular mass standard (lane 1).



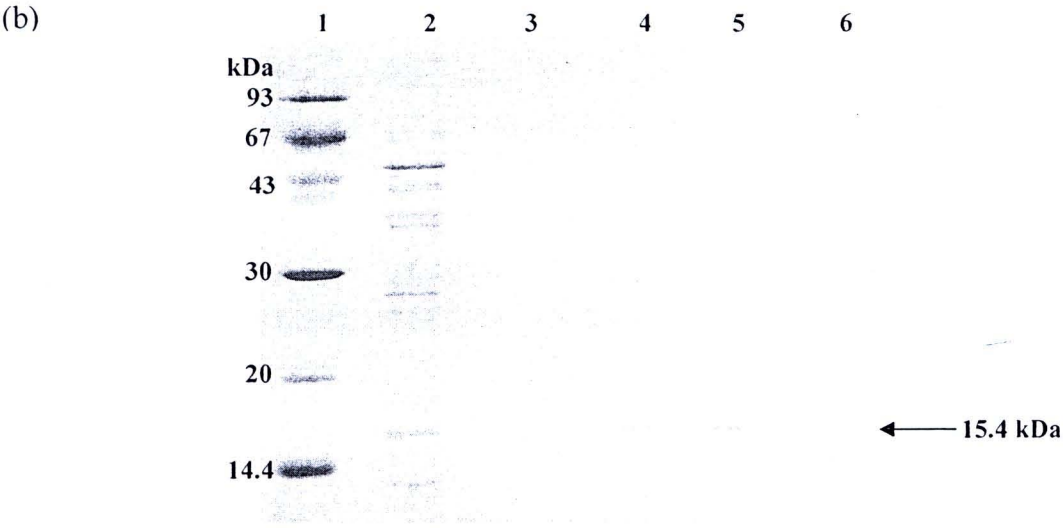


Figure 4.5 (a) Elution profile of the main fraction containing myoglobin obtained from a Sephadex G-75 column. The eluate was measured at 280 nm and 408 nm. (b) SDS-PAGE of fractions obtained from a Sephadex G-75 column; lane 2: the crude extract of myoglobin, lane 3: fraction no.16, lane 4, 5, 6: fraction no 26, 30 and 33, respectively, compared to protein low molecular mass standard (lane 1).

4.2.2 Purification of myoglobin by non-denatured polyacrylamide gel electrophoresis

Non-denatured polyacrylamide gel electrophoresis (native-PAGE) is aimed to be a simple and rapid method for protein purification. In this work, native-PAGE was developed to purify the fish myoglobin from the crude proteins sample (55-75% (w/v) saturated ammonium sulfate). Hence, the optimization conditions were needed in order to obtain suitable condition for purification of the myoglobin. The important parameters for myoglobin separation by native-PAGE were both gel pore size and applied voltage.

(1) Study of acrylamide concentration

The separation of molecules within a gel is determined by the relative pore size of the formed gel. The pore sizes of a gel depend on the total amount of acrylamide present (designated as %T). As the total amount of acrylamide increase, the pore size is decrease. The percent gels using in the separation method depend on molecular weight of the proteins. The higher gel concentration (the smaller the pore size of the gel) will be better able to separate smaller protein molecules.

Therefore, the concentrations of acryamide (%T) were optimized for myoglobin separation from the crude protein extract. Three concentrations of acrylamide, 7%T, 10%T and 15%T were selected for gel preparation. The brown color bands from each separation condition (Figure 4.6 a) were excised from the gel and extracted by grinding in the buffer (0.5 M Tris-HCl buffer, pH 8.8). The protein compositions of the extracted brown color band were determined using SDS-PAGE (Figure 4.6 b). The results showed that only one protein band (15.4 kDa) appeared when using 15%T of the native gel. This condition was thus suitable for the separation of fish myoglobin because the gel pore size was relatively small resulting in high resolution for myoglobin separation from other proteins (Figure 4.6 a). In lower gel concentrations, the large proteins could pass through the pore of the gel resulting in contamination of the brown bands on the native gel. Hence, the myoglobin separated under the acrylamide concentration of 7%T and 10%T was contaminated by larger proteins (Figure 4.6 b).

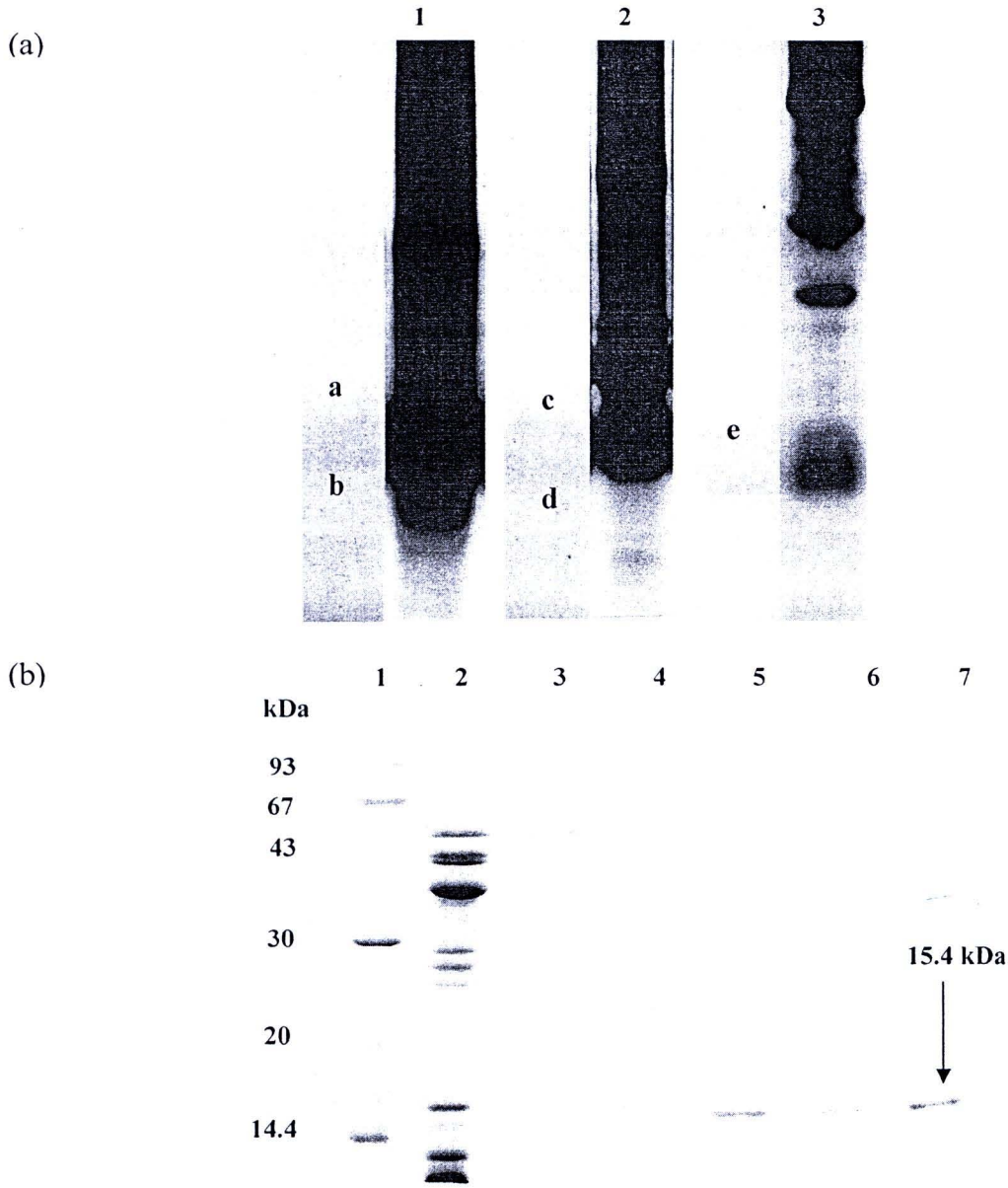


Figure 4.6 (a) Separation pattern of staining and unstaining native gel electrophoresis of crude myoglobin extract with different acrylamide gel concentrations (%T); (1): 7%T, (2): 10%T and (3): 15%T. (b) SDS-PAGE of the brown band extract solution from native-PAGE; lane 3, 4, 5, 6 and 7 obtained from the brown band; a, b, c, d and e, respectively, compared to protein low molecular mass standards (lane 1) and crude extract myoglobin (lane 2).

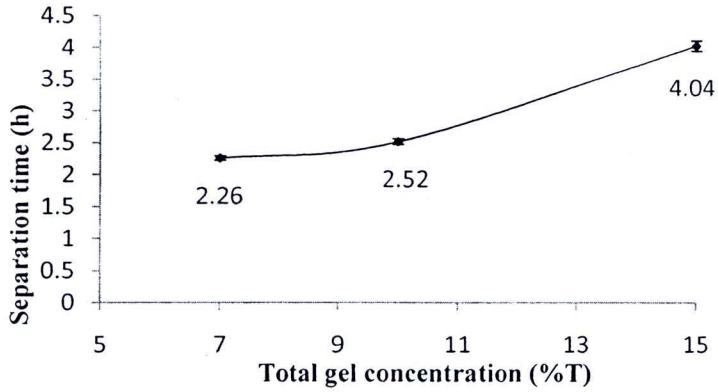


Figure 4.7 Effect of total gel concentration on separation time on the native gel electrophoresis for myoglobin purification (n=4).

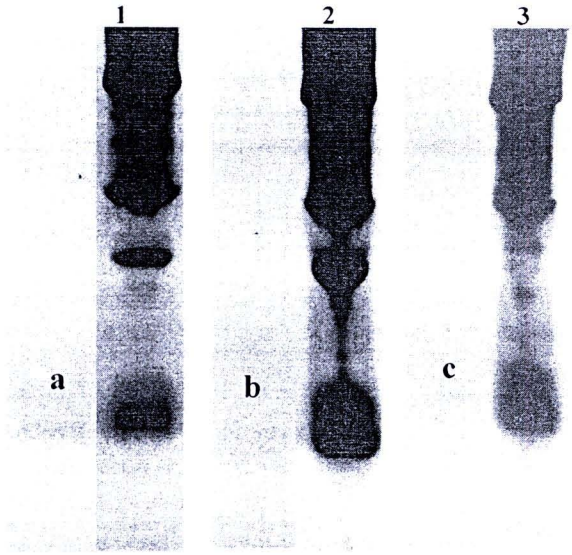
However, an increasing in the gel has affected the increasing in the separation time as shown in Figure 4.7. To improve the separation time of this electrophoretic system, the applied separation voltage was also investigated.

(2) Study of applied separation voltage

The optimization of the separation voltage was done with 15%T of acrylamide which had performed as previously described. The native PAGE with Comassie blue staining and unstaining were obtained from each applied voltage conditions as shown in Figure 4.8 a. From the result, the separation time of the native-PAGE running at 250 V was less than that of 200 V and 150 V, respectively. Because when increased in the applied separation voltage, the electric field of the procedure was increased as well, thus, proteins could move faster (Figure 4.9).

The protein composition of the brown color band obtained from each condition was determined by SDS-PAGE. All conditions could purify myoglobin from its crude proteins as shown in Figure 4.8 b. However, increased in the applied voltage affected the increaseed in temperature (Figure 4.10) resulting in the protein diffusion in the native gel (Figure 4.8 a). This result demonstrated that 15% acrylamide concentration and 250 V separation voltages were suitable for the myoglobin purification. Furthermore, the purified myoglobin obtained from native-PAGE purification method was also subjected to their property characterization including molecular mass, isoelectric point, spectral properties and partial amino acid sequences.

(a)



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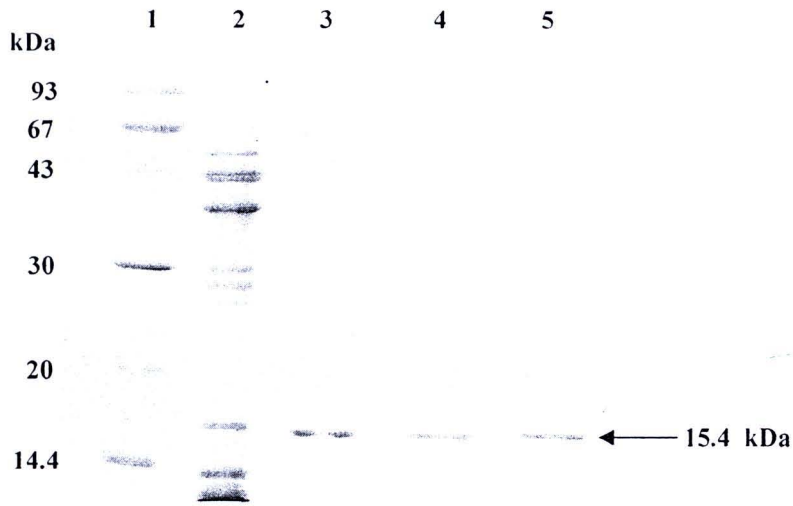


Figure 4.8 (a) Separation pattern of staining and unstaining native gel electrophoresis of crude myoglobin extract under different applied separation voltages (V); (1): 150, (2): 200 V and (3): 250 V. (b) SDS-PAGE of the brown band extract solution from native-PAGE; lane 3, 4 and 5 obtained from the brown band; a, b and c, respectively compared to protein low molecular mass standards (lane 1) and crude extract myoglobin (lane 2).

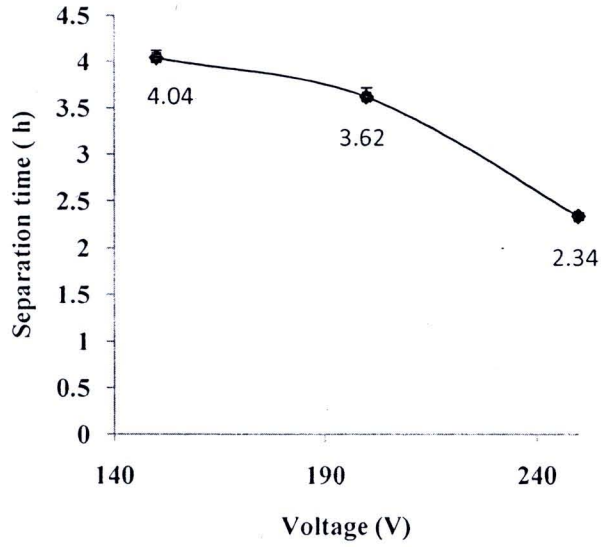


Figure 4.9 Effect of applied voltage on separation time on the native gel electrophoresis for myoglobin purification from crude protein extract (n=4).

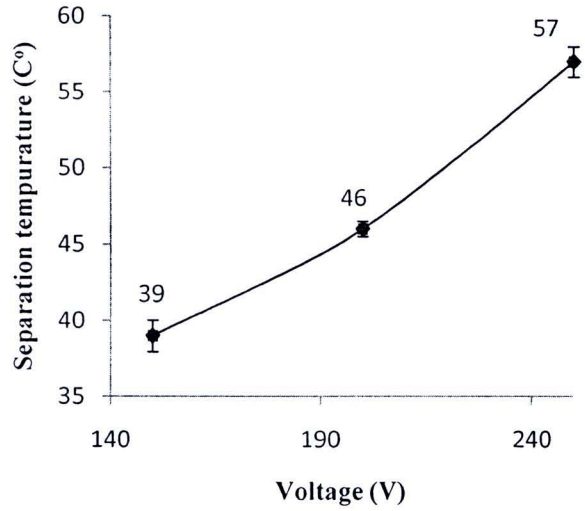


Figure 4.10 Effect of applied voltage on separation temperature on the native gel electrophoresis for myoglobin purification from crude protein extract (n=4).

4.2.3 Yield of myoglobin from the purification procedures

The yield of myoglobin from the purification procedures was indicated the purification efficiency. From Table 4.1, the content of the purified myoglobin obtained from native-PAGE was higher than that of column chromatography because the column chromatographic method possessed several steps for myoglobin purification, which the native-PAGE had only one step. Therefore, the native-PAGE method could reduce lose of the protein.

For native-PAGE method, when increased the separating voltage, the yield of the product decreased. It was due to higher diffusions of proteins under higher voltage conditions. It was found that only the slight of the brown color bands appeared under higher voltage conditions (Figure 4.8 a). Therefore, the yield of myoglobin decreased under higher voltages.

Table 4.1 Yield of the purified myoglobin obtained from column chromatography and native-PAGE ($n = 4$).

Purification method	Crude myoglobin (mg)	Purified myoglobin (mg±SD)	% Yield ±SD
Column chromatography (DEAE cellulose/ Sephadex G75)	18.54	0.22±0.06	1.2±0.3
Native-PAGE (15% gel, 150 V)	1.92	0.26±0.07	13.9±3.7
Native-PAGE (15% gel, 200 V)	1.92	0.16±0.05	8.6±2.8
Native-PAGE (15% gel, 250 V)	1.92	0.10±0.04	5.3±2.1

4.3 Characterization and identification of swamp eel myoglobin

After purification procedure, characterization and identification of fish myoglobin were necessarily carried out. In this research, fish myoglobin was characterized by using gel electrophoresis, isoelectric focusing method, UV-Visible spectrophotometry, spectrofluorometry and liquid chromatography with tandem mass spectrometry or LC-MS/MS.

4.3.1 Molecular weight and isoelectric point

The purity of fish myoglobin obtained from column chromatography and non-denatured polyacrylamide gel electrophoresis was estimated by a SDS-PAGE and MALDI-TOF mass spectrometer. Approximate molecular weight determination of the purified myoglobin can be done by SDS-PAGE (15% gel) with use of relative mobility calculation (R_f). The R_f value of the purified myoglobin was interpolated from the standard curve of log molecular weight of protein mass standards and their R_f values. The average molecular weight of fish myoglobin from five determinations was estimated about 15.43 kDa (Table 4.2). The molecular weight of this fish myoglobin was lower than that of horse heart myoglobin (Figure 4.12). It was corresponding to previous results, comparing the molecular weight of fish myoglobins were lower than those of mammalian myoglobins (Atasshi and Saplin, 1996; Fosmeir and Brown, 1976; Chaijan et. al., 2007).

MALDI-TOF mass spectrometry is an emerging technique offering promise for fast and high accurate determination of a number of protein characteristics. From the mass spectrum of the purified myoglobin, it was shown that high intensity at 15,525.18 Da was obtained (Figure 4.11). It was concluded that the molecular weight of the purified myoglobin is 15.53 kDa.

In addition, the purified myoglobin obtained from both column chromatography and native PAGE was also characterized in terms of isoelectric point by using isoelectric focusing method. The slab gel isoelectric focusing of the myoglobin obtained from both methods gave two pI values around 6.40 and 7.12 (Figure 4.13). Therefore, this protein had two isoforms.

From the results, the molecular weight and isoelectric point of the fish myoglobin obtained from both purification techniques were the same, suggesting that both methods gave the same protein.

Table 4.2 Molecular weight of swamp eel myoglobin determined by SDS-PAGE

Gel No.	Equation	R^2	$*Rf^1$	$*Rf^2$	$*MW^1$ (kDa)	$*MW^2$ (kDa)
1	y = -0.8217x + 1.8814	0.9992	0.82	0.82	16.12	16.12
2	y = -0.9405x + 1.9459	0.9925	0.81	0.81	15.04	15.04
3	y = -0.8912x + 1.8882	0.9943	0.80	0.80	14.97	14.97
4	y = -0.8833x + 1.8834	0.9935	0.76	0.76	16.10	16.10
5	y = -0.8802x + 1.894	0.9966	0.78	0.78	15.84	15.84
				$\bar{X}$±SD	15.43 ±0.56	15.43 ±0.56

Where, y = log MW of myoglobin, X= *Rf* value of myoglobin

$*Rf^1$ = *Rf* value of fish myoglobin obtained from column chromatography

$*Rf^2$ = *Rf* value of fish myoglobin obtained from native-PAGE

$*MW^1$ = Molecular weight of myoglobin obtained from column chromatography

$*MW^2$ = Molecular weight of myoglobin obtained from native-PAGE

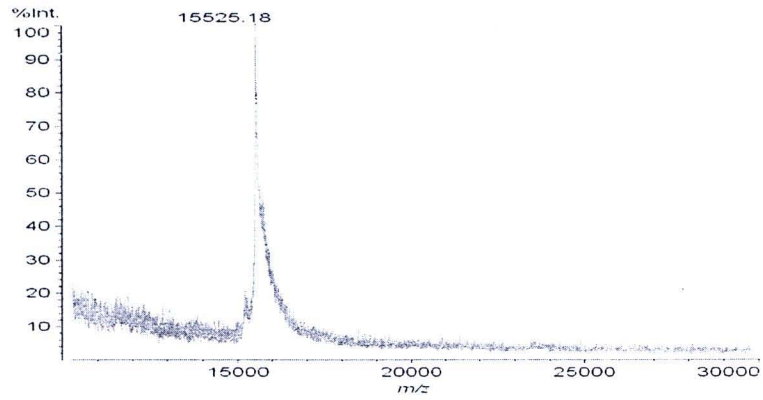


Figure 4.11 Mass spectrum of the swamp eel myoglobin obtained from a MALDI-TOF-MS

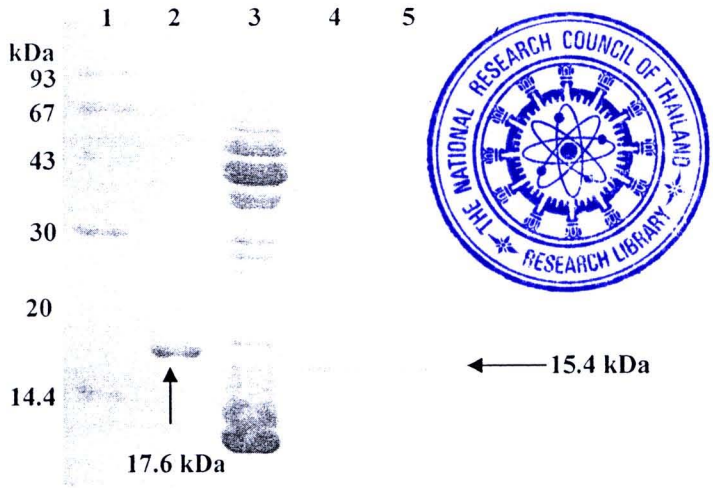


Figure 4.12 SDS-PAGE of the swamp eel myoglobin obtained from column chromatography (lane 4) and native-PAGE (lane 5) compared to protein low molecular mass standards (lane 1), crude extract myoglobin (lane 3) and horse heart myoglobin (lane 2).

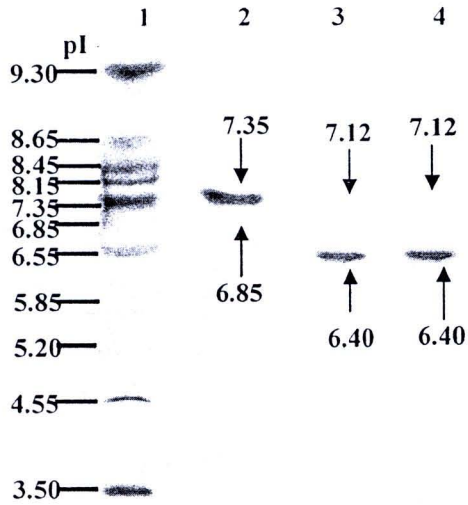


Figure 4.13 Slab isoelectric focusing gel of the swamp eel myoglobin obtained from column chromatography (lane 3) and non-denatured polyacrylamide gel electrophoresis (lane 4) compared to pI value marker (lane 1) and horse heart myoglobin (lane 2).

4.3.4 Spectral properties of myoglobin

The purified myoglobin obtained from column chromatography and non-denatured poly acrylamide gel electrophoresis were characterized in terms of absorption properties and tryptophan fluorescence quenching using both UV-Visible spectrophotometer and spectrofluorophotometer, respectively.

The UV-Visible absorption spectra of the fish myoglobin exhibited maximum absorption at 280 and 408 nm resemble to absorption spectrum of horse heart myoglobin. The purified myoglobin gave the Soret peak at 408 nm of which myoglobin had a met-form and the absorbance of protein at 280 nm depends on the content of tryptophan, tyrosine and cysteine residues (Figure 4.14).

The study of tryptophan fluorescence intensity of the fish myoglobin, horse heart myoglobin and bovine serum albumin were done by a spectrofluorophotometer. The fluorescence intensity of the hemoprotein is quenched by the heme group, it is interesting to confirm that the heme was existed in fish myoglobin. From Figure 4.15, it is shown that the fluorescence intensity of the fish myoglobin and horse heart myoglobin at 325 nm is lower than that of bovine serum albumin. It was confirmed that the purified myoglobin is the hemoprotein.

Furthermore, tryptophan fluorescence is widely used as a tool to monitor structural changes in proteins and to make interferences regarding local structure and dynamics. Tryptophan fluorescence can be used to follow protein folding because their properties are sensitive to their environment which changes when protein folds/unfolds. In the native folded state, tryptophan and tyrosine are generally located within the core of the protein, whereas in a partially folded or unfolded state, they become exposed to solvent. In a hydrophobic environment tryptophan and tyrosine have high quantum yield and therefore high fluorescence intensity. In contrast, in a hydrophilic environment (exposed to solvent) their quantum yield decreased leading to low fluorescence intensity. From the native-PAGE, the fluorescence intensity of the purified myoglobin obtained from high voltage conditions were lower than that of low voltage conditions because when the applied voltage increased, the protein was unfolded. Therefore, fluorescence intensity of the purified myoglobin obtained from native gel electrophoresis was lower than that of the purified myoglobin obtained from column chromatography.

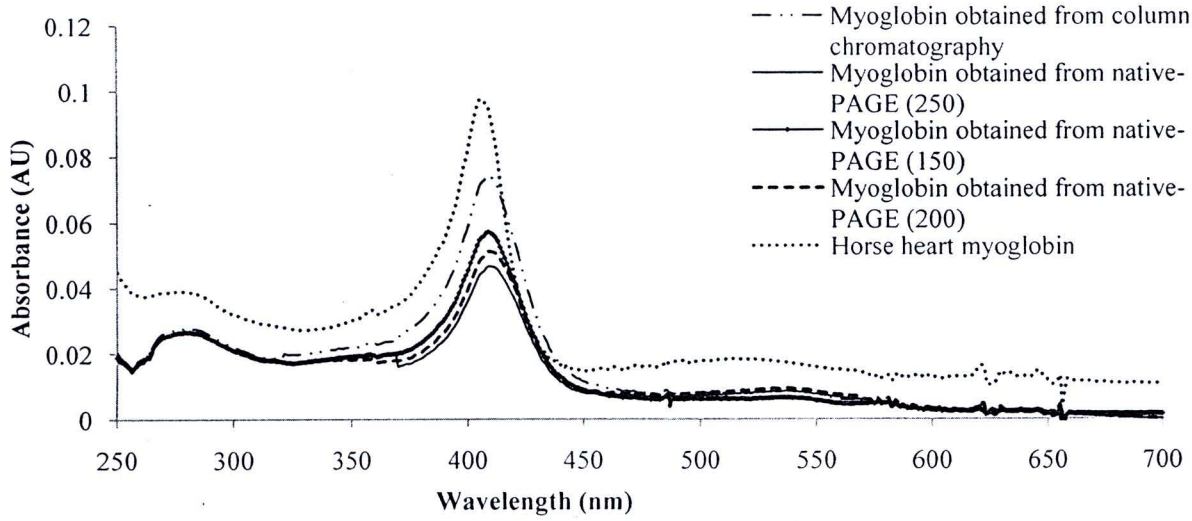


Figure 4.14 Absorption spectra of swamp eel myoglobin obtained from both column chromatography and native-PAGE compared to horse heart myoglobin.

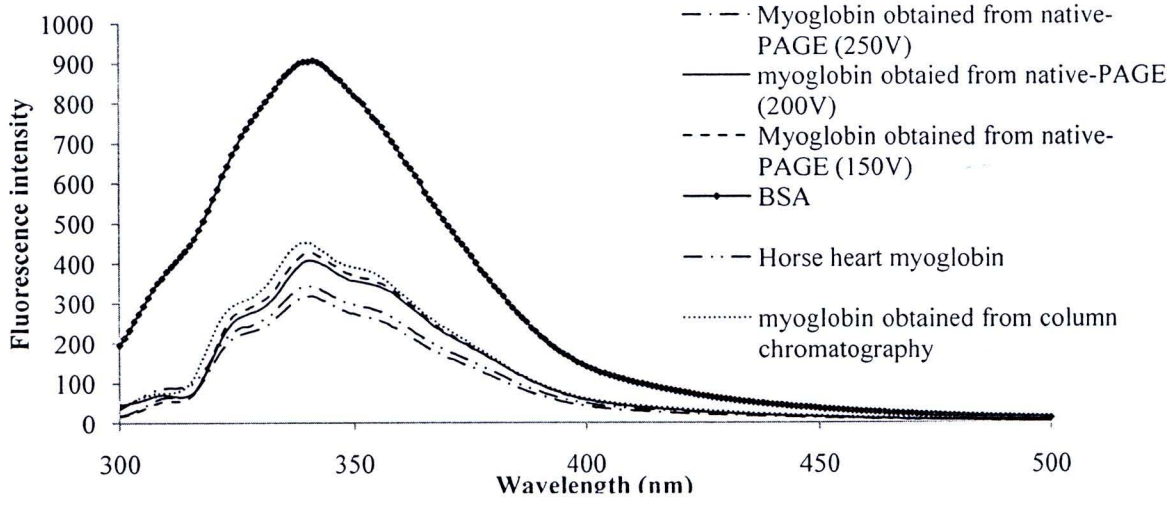


Figure 4.15 Tryptophan fluorescence spectra of swamp eel myoglobin obtained from both column chromatography and native-PAGE compared to horse heart myoglobin and bovine serum albumin (BSA).

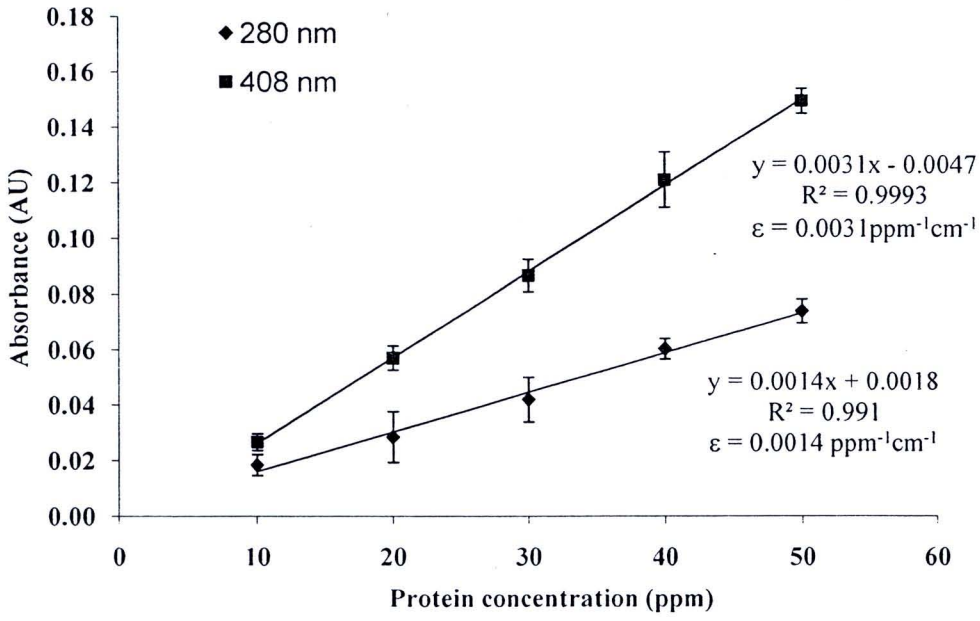
4.3.4 Molar extinction coefficient

Purified myoglobin obtained from column chromatography was used to determined extinction coefficient of this protein. Extinction coefficient of swamp eel myoglobin was calculated from the slope of the straight curve by plotting the concentration of protein versus absorbance (Figure 4.16). From the results, the extinction coefficient (ε) of fish myoglobin was found to be 2.22×10^{4} $M^{-1}cm^{-1}$, 1.4×10^{-3} $ppm^{-1}cm^{-1}$ at 280 nm and 4.80×10^{4} $M^{-1}cm^{-1}$, 3.1×10^{-3} $ppm^{-1}cm^{-1}$at 408 nm. These coefficients were used to determine the myoglobin concentration by using Lambert-Beer's law from the equation of

$$A = \varepsilon bc$$

Where, A = absorbance of the sample measured, b = thickness of the sample cell, c = concentration of myoglobin and ε = extinction coefficient constant.

(a)



(b)

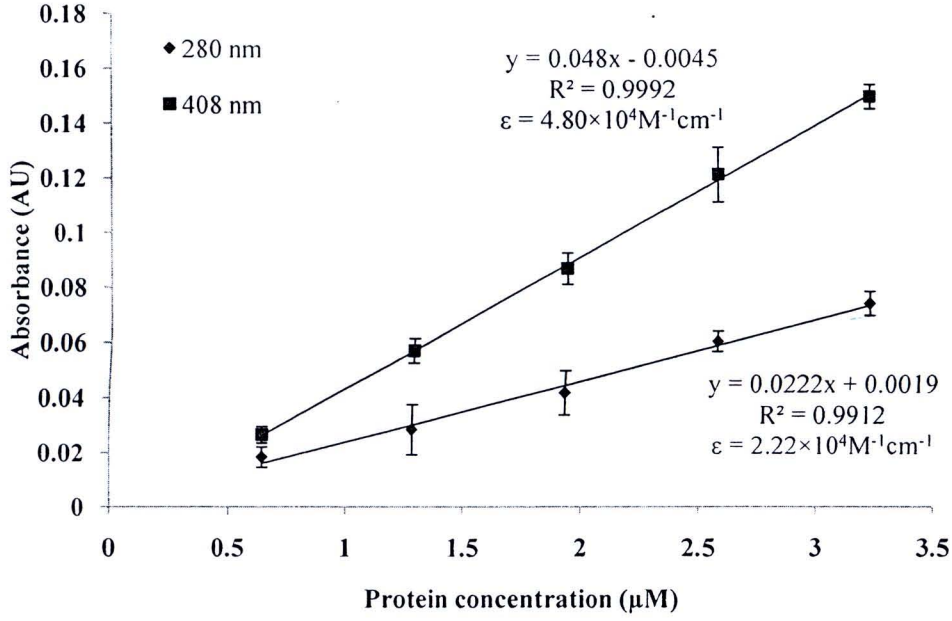


Figure 4.16 Beer's law plot of swamp eel myoglobin in 50 mM Tris-HCl buffer (pH 6.8). The extinction coefficient of this protein was calculated in unit of $ppm^{-1}cm^{-1}$ (a) and $M^{-1}cm^{-1}$ (b).

4.3.5 Partial peptide sequence of swamp eel myoglobin

All proteins are made up from the same set of 20 standard amino acids. The sequence of proteins was largely effective for differences in structure and function of individual proteins. Amino acid composition of myoglobin was one of the characteristic properties. This sequence relates to the structural, functional and physical properties of myoglobin. In this research, the purified myoglobin obtained from native-PAGE (15%T, 250V) was used to determine their partial peptide sequences using LC-MS/MS. After purification of myoglobin by native-PAGE, the brown color band extract was separated on SDS-PAGE. This SDS gel showed the single band with a molecular weight of 15.4 kDa. The protein band was excised from the gel and then determined using in-gel digestion with trypsin and analyzed the peptide fragments by LC-MS/MS performed at the Genome Institute, BIOTEC, Pathumthani, Thailand. From the results, six partial peptide sequence tags from LC-MS/MS were obtained as shown in Table 4.3. Each of the peptide fragments was identified by using Turbo SEQUEST Algorithm in BioWorks^{TM} 3.1SR1 software package (Thermo Electron) and nr.fasta database. The identified peptide fragments were further evaluated using cross-correlation number (Xcorr), the Xcorr values are higher for well-matched peptide sequences. The fragments which have high Xcorr values were identical to myoglobin from some marine fish species; sea raven *(Hemitripterus americanus)*, yellowfin tuna (*Thunnus albacores*) and blue marlin (*Makaira nigricans*). Therefore, it was concluded that the purified protein was the fish myoglobin. In addition, the partial peptide sequence tags obtained from the fish myoglobin are homologous to full amino acid sequence of marine fish species in the ExPASy Proteomics Server as shown in Table 4.4. From this data, myoglobin of sea raven was the highest amino acid matching to the sequence tags from LC-MS/MS, those of yellowfin tuna and blue marlin were less homology. Therefore, it supposes that some structural and functional properties of swamp eel myoglobin were similar to those of marine fish myoglobins.

Table 4.3 Identification of protein bands (15.4 kDa) on SDS-PAGE obtained from the purified myoglobin using LC-MS/MS and database search

Sequence tags from LC-MS/MS	Cross-correlation	Protein identification
AKGSHAAILKPLANSHATK	5.94	Myoglobin from sea raven *(Hemitripterus americanus)*, Yellowfin tuna (*Thunnus albacores*) and Blue marlin (*Makaira nigricans*)
GSHAAILKPLANSHATK	4.55	
LFTEHPETQK	3.19	
LFTEHPETQKLFPK	3.16	
HKIPINNFR	2.44	
IPINNFR	2.20	

Table 4.4 Comparisons of partial amino acid sequences obtained from LC-MS/MS and full amino acid sequence of marine fish myoglobins from ExPASy_Proteomics Server. All amino acids corresponding to the sequence of fish myoglobins from database search are highlighted by gray color.

Myoglobin source	Protein sequence alignment	% matching
Blue marlin	MADFEMVLKH WGPVEADYAT HGNLVLTRLF TEHPETQKLF PKFAGIAKAD MAGNAAISAH GATVLKKLGE LLKAKGSHAA IIKPMANSHA TKHKIPIKNF ELISEVIGKV MHEKAGLDAA GQKALKNVMT TIIADIEANY KELGFTG	25.33
Sea raven	LVLKCWGPVE ADYAAYGSLV LTRLFTEHPD TQKLFPKFAG IAQGDMAADA RKLGELLKAK GSHAAILKPL ANSHATKHKI PINNFRLITE VIGKVMGEKT GLDAAGQQAL RNVMAIVVAD MEADYKLLGF TG	30.09
Yellowfin tuna	MADFDAVLKC WGPVEADYTT MGGLVLTRLF KEHPETQKLF PKFAGIAQAD IAGNAAISAH GATVLKKLGE LLKAKGSHAA ILKPLANSHA TKHKIPINNF KLISEVLVKV MHEKAGLDAG GQTALRNVMG IIIADLEANY KELGFSG	27.21

4.3.6 Autoxidation rate constant of swamp eel myoglobin

The oxygen affinity of myoglobin is an important function of myoglobin properties. This characteristic was supposed to be related to oxygen storage function of myoglobin. Autoxidation rate of myoglobin was characterized by UV-Visible absorption spectrum of myoglobin in the region of 475-700 nm. In the oxy-form of myoglobin, ferrous ion in conjunction to dioxygen exhibits the absorption maxima around 542 (β peak) and 581 nm (α peak). After myoglobin released oxygen molecule, the spectrum of myoglobin (deoxy-form) changed to the single peak of about 556 nm. Moreover, the ferrous ion in myoglobin structure was oxidized to ferric ion and the spectrum of myoglobin (met-form) exhibited the absorption maxima about 505 and 630 nm. The autoxidation rate of myoglobin thus reflects the changing rate of oxymyoglobin to metmyoglobin, and it was investigated by using UV-Visible spectrometry at 581 nm. The rate constant (k_{obs}) was calculated from the change of spectrum using first order plot of Time (h) *vs* $(MbO_2)_t/(MbO_2)_0$).

The autoxidation rate of myoglobin from swamp eel fish was determined compared with that of horse heart myoglobin. The oxymyoglobin was prepared by adding sodium dithionite in myoglobin solution in order to convert ferric ion to ferrous ion. Then, the myoglobin containing ferrous ion (deoxymyoglobin) was bubbled by oxygen gas. This protein solution changed from the red-brown color to bright red color indicating myoglobin binding oxygen (oxy-form). The spectral changes of fish myoglobin in each form are shown in Figure 4.17. Oxymyoglobin was used to determine autoxidation rate constant at 37°C in 0.2 mM phosphate buffer pH 6.8 by UV-Visible spectrophotometry. It gave the visible band at 581 nm (α peak) for calculating k_{obs} from the slope of the first-order plot. After incubation myoglobin at 37°C, the absorption at α peak decreased because the oxymyoglobin released its oxygen molecule. The absorption changes at 581nm at time interval of this myoglobin were shown in Figure 4.18. The observed first-order rate constants (k_{obs}) for the autoxidation of this fish myoglobin and horse heart myoglobin were shown in Figure 4.19. The average k_{obs} values obtained from this fish myoglobin and horse heart myoglobin were found to be 1.42 h^{-1} and 1.08 h^{-1}, respectively (Table 4.5). From the results, oxygen in fish myoglobin could be easily released compared with horse heart myoglobin because the swamp eel myoglobin had lower oxygen affinity than horse

heart myoglobin. From this reason it also suggested that the heme pocket of the swamp eel myoglobin was more open than that of horst heart myoglobin. It was corresponded to previous report of Nichols and Weber, 1989 that the autoxidation rate of fish myoglobin was more susceptible than that of mammalian myoglobins.

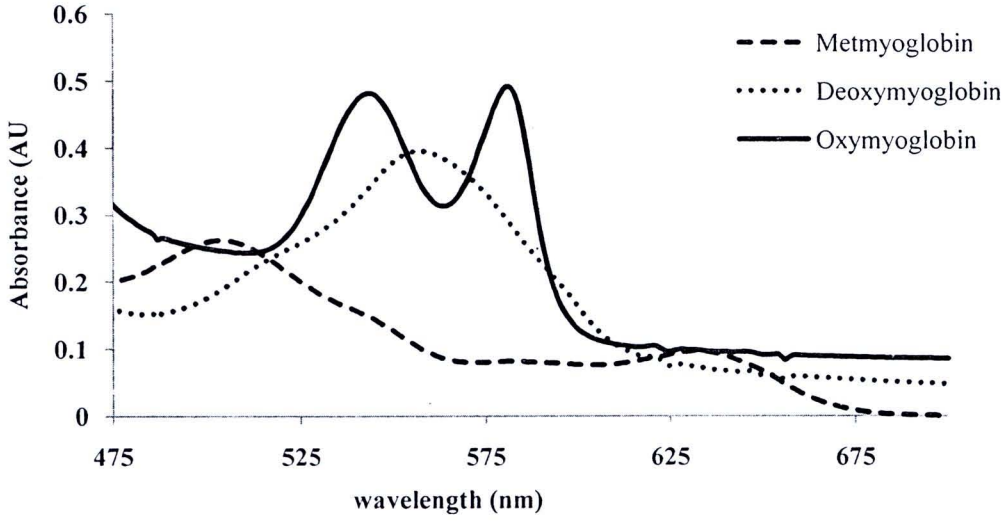


Figure 4.17 Absorption spectra of metmyoglobin (Fe III), deoxymyoglobin (Fe II) and oxymyoglobin (Fe II-O_2).

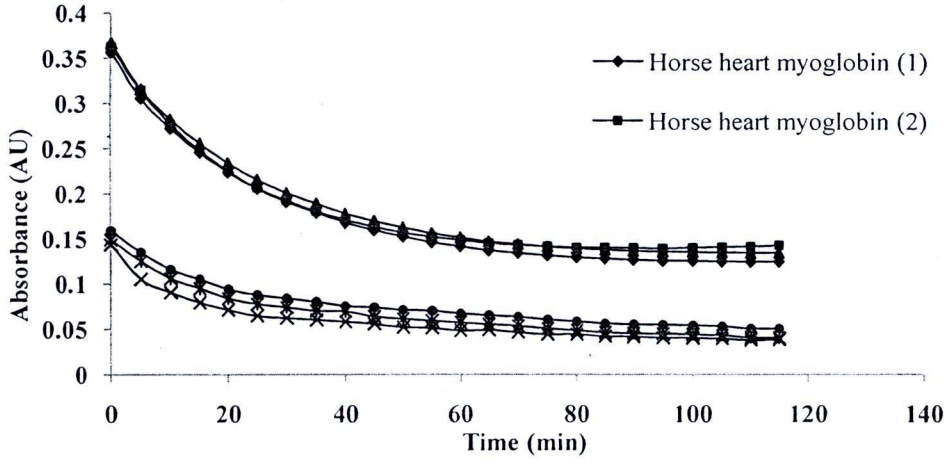


Figure 4.18 Absorbance changes (A_{581}) with time for the autoxidation of the swamp eel MbO_2 and horse heart MbO_2 at 37°C in 0.2 mM phosphate buffer (pH 6.8).

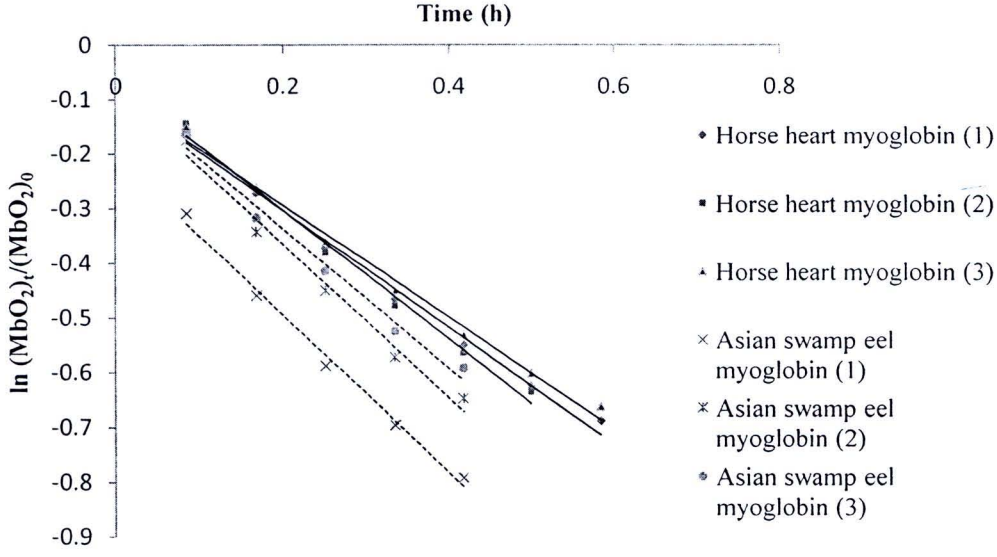


Figure 4.19 First-order plots for the autoxidation of MbO_2 prepared from Asian swamp eel myoglobin and horse heart myoglobin at 37°C in 0.2 mM phosphate buffer (pH 6.8).

Table 4.5 Observed first-order rate constants (k'_{obs}) obtained from Asian swamp eel myoglobin and horse heart myoglobin at 37°C in 0.2 mM phosphate buffer (pH 6.8).

Sample	No.	Equation	R^2	Slope	k'_{obs} (h)	X±SD
Asian swamp eel	1	y = -1.443x - 0.2066	0.9919	-1.443	1.443	
myoglobin	2	y = -1.4084x - 0.084	0.9834	-1.4084	1.4084	1.42 ± 0.018
	3	y = -1.4175x - 0.0584	0.9896	-1.4175	1.4175	
Horse heart	1	y = -1.067x - 0.0903	0.9909	-1.067	1.067	
myoglobin	2	y = -1.1773x - 0.0676	0.9901	-1.1773	1.1773	1.08 ± 0.079
	3	y = -1.0225x - 0.0893	0.9903	-1.0225	1.0225	

(n=3)