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APPENDICES

APPENDIX A
ELECTROPHORETIC METHODS

A1. Sodium dodeccyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
(Laemmli, 1970)

Separating gel solution, 15%T

Acrylamide/Bisacrylamide (30%T)	2.0	mL
1.5 M Tris-HCL, pH 8.8	1.0	mL
Deionized water	0.940	mL
10% SDS	40.0	μL
10% Ammonium persulfate	17.5	μL
TEMED	2.0	μL

Stacking gel solution, 4%T

Acrylamide/Bisacrylamide (30%T)	0.265	mL
1.5 M Tris-HCL, pH 8.8	0.5	mL
Deionized water	1.240	mL
10% SDS	20	μL
10% Ammonium persulfate	12.5	μL
TEMED	6.25	μL

Where, %T= Total acrylamide concentration

A2. Non-denatured polyacrylamide gel electrophoresis (Native PAGE)

Separating gel solution, 7%T

Acrylamide/Bisacrylamide (30%T)	0.93	mL
1.5 M Tris-HCL, pH 8.8	1.0	mL
Deionized water	2.05	mL
10% Ammonium persulfate	17.5	µL
TEMED	2.0	µL

Separating gel solution, 10%T

Acrylamide/Bisacrylamide (30%T)	1.33	mL
1.5 M Tris-HCL, pH 8.8	1.0	mL
Deionized water	1.65	mL
10% Ammonium persulfate	17.5	µL
TEMED	2.0	µL

Separating gel solution, 15%T

Acrylamide/Bisacrylamide (30%T)	2.0	mL
1.5 M Tris-HCL, pH 8.8	1.0	mL
Deionized water	0.980	mL
10% Ammonium persulfate	17.5	µL
TEMED	2.0	µL

Stacking gel solution, 4%T

Acrylamide/Bisacrylamide (30%T)	0.265	mL
1.5 M Tris-HCL, pH 8.8	0.5	mL
Deionized water	1.260	mL
10% Ammonium persulfate	12.5	µL
TEMED	6.25	µL

Where, %T= Total acrylamide concentration

A.3 Isoelectric focusing (IEF)

Gel solution, 5.5%T

Acrylamide/Bisacrylamide (30%T)	0.265	mL
Ampholyte pH 3.5-10	0.105	mL
Deionized water	1.635	mL
10% Ammonium persulfate	12.5	µL
TEMED	6.25	µL

Where, %T= Total acrylamide concentration

APPENDIX B
CHROMATOGRAPHIC MEDIA

B1. Sephadex G75

Sephadex G-75 is highly specialized gel filtration and chromatographic media. These composed of macroscopic beads synthetically derived from the polysaccharide, dextran. The organic chains are cross-linked to give a three dimensional network having functional ionic groups attached by ether linkages to glucose units of the polysaccharide chains.

Table B1 Technical information of Sephadex G-75

Factor	Technical specification
Composition	Cross-linked dextran
Particle size	Dry, min. 80% volume share between 40-120 μm, wet (in o.15 M NaCl), 55-310 μm
Fractionation range, globular protein	3×10^3 - 7×10^4 Da
pH stability	2-13
CIP stability	2-13
Pressure and flow spec	U_{max} min 90 cm/h, bed height 10 cm, column 5 cm i.d.

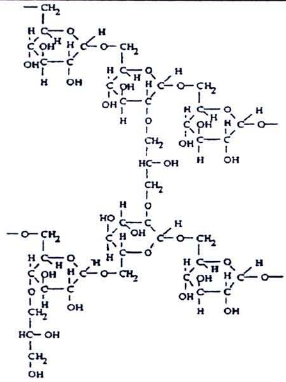


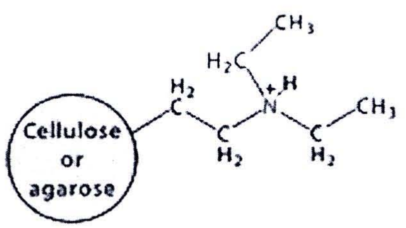
Figure B1. Partial structure of Sephadex

B2. DEAE cellulose

DEAE-cellulose is a weak ion exchanger based on the diethylaminoethyl (DEAE) tertiary amine functional group which allows fast flow rates especially after fine removal and is suitable for negative charged biopolymers.

Table B2 Technical information of DEAE-cellulose

Factor	Technical specification
Type of ion exchanger	Weak cation exchanger
Functional group	Diethylaminoethyl
Nomal pH range	2-9.5
Small ion capacity (meq/dg) (mg/dg)	 0.88-1.08 700b
Protein capacity (mg/ml) bed volume	60



Diethylaminoethyl
(DEAE) group
(protonated form)

Figure B2 Structure of DEAE-cellulose

APPENDIX C
METHODS FOR CHARACTERIZATION OF MYOGLOBIN USING LC-MS/MS

C1 Spot handing method parameters

Table C1 Standard method for Coomassie stained gel

Method	Category	Parameter	Recommended	Unit
Spot picking	Spot picking	Picking head diameter	1.4/2.0	mm
		Aspirate	40	µl
		Dispense	Water,150	µl
		Rinse picker head	No	-
		Aspirate	200	µl
		Prime	Water	
Digestion	Mp 1 Preparation Step 1	Dispense reagent	Ambic 50 mM/ MeOH 50%, 100	µl
		Incubate	Hotel,20	min
		Aspirate reagent	150	µl
		Rinse needle	NO	
		Dispense reagent	Ambic 50 mM/MeOH 50%, 100	µl
	MP 1 Preparation Step 2	Incubate	Hotel,20	min
		Aspirate reagent	150	µl
		Rinse needles	NO	
		Dispense reagent	CAN 75%,100	µl
	MP1 Preparation Step 3	Incubate	Hotel,10	min
		Aspirate reagent	150	µl
		Rinse needles	NO	
	MP 1 Drying		Selected,15	min

Table C1 Standard m€ethod for Coomassie stained gel (Cont.)

Method	Category	Parameter	Recommended	Unit
Digestion	Enzyme addition	Transfer enzyme priming	Ambic 20 mM	µl
		Transfer enzyme priming	Trypsin/Ambic 20 mM, 10	µl
	Digestion	Incubate	Incubator, 60	min
	Extraction	Number of step 2		
	Extraction 1	Dispense liquid in MP 1	ACN50%/TFA 0.1%,40	µl
		Incubate	Hotel,20	min
		Transfer MP 1 to MP 2	Selected,80	µl
	Extraction 2	Dispense liquid in MP 1	ACN50%/TFA 0.1%, 40	µl
		Incubate	Hotel,20	min
		Transfer MP 1 to MP 2	Selected, 60	µl
	MP 2 Drying		90	min
Spotting	Procedure		Dissolve in matrix	
	Liquids	Priming	ACN50%/TFA 0.5%	µl
		Matrix	4-HCCA mix, 3.0	µl
		Sample	0.3	
	Sample Dissolving	Add liquid in well	Matrix	strokes
		Mix in well	5	
			3.0	µl
	spotting	First sequence	Dissolve in matrix	

C2. Liquid chromatography coupled with tandem mass spectrometry

LC-MS/MS model Finnigan LTQ Linear Ion Trap mass Spectrometer was used to determine the amino acid sequence. Protein identification was performed using the Turbo SEQUEST algorithm in the Bio Works ™ 3.1SR1 software package (Thermo Electron) and nr.fasta database. The identified peptides were further evaluated using charge state versus cross-correlation number (Xcorr). The criteria for positive identification of peptides was Xcorr > 1.5 for singly charged ions, Xcorr > 2.0 for doubly charged ion, and Xcorr>2.5 for triply charged ions.

Table C2 LC separation and MS analysis conditions

LC separation	**Conditions**
HPLC system	Finnigan Surveyor ™MS pump with a flow splitter
Column	0.18 × 100 mm C18 (Themo Electron)
Flow rate	200μl/min
Mobile phase	A: Water with 0.1% formic Acid
	B: Acetonitrile with 0.1% formic acid
Gradients	2-60%B in 20 min
	65-80%B in 5 min and hold 5 min,
	80-2%B in 2 min
MS analysis	**Conditions**
Mass Spectrometer	Finnigan LTQ
Ionization mode	NanoSpray, positive ion
Capillary temperature	200 °C
Spray needle voltage	1.8 KV
Mass range	400-1,600 m/Z
Scan sequence	Full-scan MS, MS/MS scan
Acquisition modes	Normal, DATA dependent ™ and Dynamic Exclusion ™
Database	

APPENDIX D

AMINO ACID SEQUENCES OF MYOGLOBINS FROM ANIMALS

Table D1.1 Amino acid sequences of marine fish myoglobins; North Pacific bluefin tuna, Bluefin tuna, Bigeye tuna and Yellowfin tuna.

	10	20	30	40	50	60
North Pacific bluefin tuna	MADFDAVLKC	WGPVEADYTT	IGGLVLTRLF	KEHPETQKLF	PKFAGIAQAD	IAGNAAVSAH
Bluefin tuna	MADFDAVLKC	WGPVEADYTT	IGGLVLTRLF	KEHPETQKLF	PKFAGIAQAD	IAGNAAVSAH
Bigeye tuna	MADFDAVLKC	WGPVEADYTT	IGGLVLTRLF	KEHPETQKLF	PKFAGIAQAD	IAGNAAVSAH
Yellowfin tuna	MADFDAVLKC	WGPVEADYTT	MGGLVLTRLF	KEHPETQKLF	PKFAGIAQAD	IAGNAAISAH
	70	80	90	100	110	120
North Pacific bluefin tuna	GATVLKKLGE	LLKAKGSHAA	ILKPLANSHA	TKHKIPINNF	KLISEVLVKV	MHEKAGLDAG
Bluefin tuna	GATVLKKLGE	LLKAKGSHAA	ILKPLANSHA	TKHKIPINNF	KLISEVLVKV	MHEKAGLDAG
Bigeye tuna	GATVLKKLGE	LLKAKGSHAA	ILKPLANSHA	TKHKIPINNF	KLISEVLVKV	MHEKAGLDAG
Yellowfin tuna	GATVLKKLGE	LLKAKGSHAA	ILKPLANSHA	TKHKIPINNF	KLISEVLVKV	MHEKAGLDAG
	130	140	147			
North Pacific bluefin tuna	GQTALRNVMG	IIIADLEANY	KELGFSG			
Bluefin tuna	GQTALRNVMG	IIIADLEANY	KELGFSG			
Bigeye tuna	GQTALRNVMG	IIIADLEANY	KELGFSG			
Yellowfin tuna	GQTALRNVMG	IIIADLEANY	KELGFSG			

Table D1.2 Amino acid sequences of marine fish myoglobins; Sea Raven, Blue Marlin, Albacore and Chub Mackerel.

	10	20	30	40	50	60
Sea Raven	LVLKCWGPVE	ADYAAYGSLV	LTRLFTEHPD	TQKLFPKFAG	IAQGDMAADA	GISAHGATVL
Blue Marlin	MADFEMVLKH	WGPVEADYAT	HGNLVLTRLF	TEHPETQKLF	PKFAGIAKAD	MAGNAAISAH
Albacore	MADFDAVLKC	WGPVEADYTT	IGGLVLTRLF	KEHPDTQKLF	PKFAGIAQAD	LAGNAAISAH
Chub Mackerel	MADFDAVLKF	WGPVEADYDK	IGNMVLTRLF	TEHPDTQKLF	PKFAGIGLGD	MAGNAAISAH

	70	80	90	100	110	120
Sea Raven	RKLGELLKAK	GSHAAILKPL	ANSHATKHKI	PINNFRLITE	VIGKVMGEKT	GLDAAGQQAL
Blue Marlin	GATVLKKLGE	LLKAKGSHAA	IIKPMANSHA	TKHKIPIKNF	ELISEVIGKV	MHEKAGLDAA
Albacore	GATVLKKLGE	LLKAKGSHAS	ILKPMANSHA	TKHKIPINNF	KLISEVLVKV	MQEKAGLDAG
Chub Mackerel	GATVLKKLAE	VLKAKGNHAG	IIKPLANSHA	TKHKIAINNF	KLITEIIVKV	MQEKAGLDAG

	130	140	147
Sea Raven	RNVMAIVVAD	MEADYKLLGF	
Blue Marlin	GQKALKNVMT	TIIADIEANY	KELGFTG
Albacore	GQKALKNVMT	TIIADIEANY	KELGFTG
Chub Mackerel	GQTALRNVMG	VFIADMDANY	KELGFSG

Table D1.3 Amino acid sequences of marine fish myoglobins; Sard, Green puffer, Red sea bream and crystal darter.

	10	20	30	40	50	60
Sard	MADFDAVLKF	WGPVEADYTS	HGGLVLTRLF	KEHPETQKLF	PKFTGIAQAD	MAGNAAISAH
Green puffer	MGDFDMVLKF	WGPVEADYSA	HGGMVLTRLF	TENPETQQLF	PKFVGIAQSE	LAGNAAVSAH
Red sea bream	MDDFEKVLKF	WGPVEADYNA	HGGLVLNRLF	MERPETQQLF	PKFVGIAPGD	LAGNAAVSAH
crystal darter	LFPKFAGIAQ	SDLAGNAAIS	AHGATVLKKL	GELLRAKGNH	AAILKPLANT	HATKHKIPIN
	70	80	90	100	110	120
Sard	GATVLKKLGE	LLKAKGNHAA	ILKPMANSHA	TKHKIPINNF	KLISEIIVKV	MQEKAGMDAG
Green puffer	GATVLKKLGE	LLKAKGNHAA	ILQPLANSHA	TKHKIPIKNF	KLIAEVIGKV	MAEKAGLDTA
Red sea bream	GATVLKKLAE	LLKAKGDHAA	ILKPMATSHA	TKHKIPLANF	ELMTEIIAKV	MEEKAGLDAA
crystal darter	NFRLISEVVV	KVMVEKAGLD	A			
	130	140	147			
Sard	GQQALRNVMA	AVIADLEANY	KELGFSG			
Green puffer	GQQALRNIMA	TIIADIDATY	KELGFS			
Red sea bream	GQQALRNVMA	VIIADMDVTY	KELGFKA			

Table D1.3 Amino acid sequences of marine fish myoglobins; Crocodile icefish, Unicorn icefish, Black rockcod and Humped rockcod.

	10	20	30	40	50	60
Crocodile icefish	MADFDMVLKC	WGPVEADHAT	HGSLVLTRLF	TEHPETLKLF	PKFAGIAHGD	LAGDAGVSAH
Unicorn icefish	MADFDMVLKC	WGPVEADHAT	HGSLVLTRLF	TEHPETLKLF	PKFAGIAHGD	LAGDAGVSAH
Black rockcod	MADFDMVLKC	WGPMEADYAT	HGGLVLTRLF	TEHPETLKLF	PKFAGIAHGD	LAGDAGVSAH
Humped rockcod	MADFDMVLKC	WGPVEADYTT	HGSLVLTRLF	TEHPETLKLF	PKFAGIAHGD	LAGDAGVSAH
	70	80	90	100	110	120
Crocodile icefish	GATVLKKLGD	LLKARGGHAA	LLKPLSSSHA	TKHKIPIINF	TLIAEVIGKV	MEEKAGLDAA
Unicorn icefish	GATVLKKLGD	LLKARGGHAA	LLKPLSSSHA	TKHKIPIINF	KLIAEVIGKV	MEEKAGLDAA
Black rockcod	GATVLNKLGD	LLKARGAHAA	LLKPLSSSHA	TKHKIPIINF	KLIAEVIGKV	MEEKAGLDAA
Humped rockcod	GATVLNKLGD	LLKARGAHAA	LLKPLSSSHA	TKHKIPIINF	KLIAEVIGKV	MEEKAGLDAA
Crocodile icefish	130	140	147			
Unicorn icefish	GQTALRNVMA	IIITDMEADY	KELGFTE			
Black rockcod	GQTALRNVMA	IIITDMEADY	KELGFTE			
Humped rockcod	GQTALRNVMA	VIIADMEADY	KELGFTE			
	GQTALRNVMA	VIIADMEADY	KELGFTE			

Table D2 Amino acid sequences of fresh water fish myoglobins; Common carp and Goldfish.

	10	20	30	40	50	60
Common carp	MADYERFLKC	WGAIEADYAG	HGGEVLTRLF	KEHPDTLKLF	PKFKGIPQSE	LAGDTLVASH
Goldfish	MADHELVLKC	WGVVEADFEG	TGGEVLTRLF	KQHPETQKLF	PKFVGIAQSD	LAGNAAVNAH
	70	80	90	100	110	120
Common carp	GATVLKKLGE	LLRAKGDHAA	ILQPLATTHA	NKHKIALNNF	RLITEVLVKV	MAEKAGLDTA
Goldfish	GATVLKKLGE	LLKARGDHAA	ILKPLATTHA	NKHKIALNNF	RLITEVLVKV	MAEKAGLDAA
	130	140	147			
Common carp	GQGALKRVMD	CIIRDIDRYY	KEIGFAG			
Goldfish	GQTALRKVME	AVIGDIDTYY	KEFGFAG			

Table D3.1 Amino acid sequences of mammalian myoglobins;. Egyptian fruit bat, Megaleia rufa, Brown-capped capuchin and Australian echidna.

	10	20	30	40	50	60
Egyptian fruit bat	MGLSDGEWQL	VLNVWGKVEA	DIPGHGQEVL	IRLFKGHPET	LEKFDKFKHL	KSEDEMKASE
Megaleia rufa	MGLSDGEWQL	VLNIWGKVET	DEGGHGKDVL	IRLFKGHPET	LEKFDKFKHL	KSEDEMKASE
Brown-capped capuchin	MGLSDGEWQL	VLNVWGKVEA	DIPSHGQEVL	ISLFKGHPET	LEKFDKFKHL	KSEDEMKASE
Australian echidna	MGLSDGEWQL	VLKVWGKVET	DITGHGQDVL	IRLFKTHPET	LEKFDKFKHL	KTEDEMKASE
	70	80	90	100	110	120
Egyptian fruit bat	DLKKHGATVL	TALGGILKKK	GQHEAQLKPL	AQSHATKHKI	PVKYLEFISE	VIIQVLQSKH
Megaleia rufa	DLKKHGITVL	TALGNILKKK	GHHEAELKPL	AQSHATKHKI	PVQFLEFISD	AIIQVIQSKH
Brown-capped capuchin	ELKKHGATVL	TALGGILKKK	GQHEAELKPL	AQSHATKHKI	PVKYLEFISD	AIVHVLQKKH
Australian echidna	DLKKHGGVVL	TALGSILKKK	GQHEAELKPL	AQSHATKHKI	SIKFLEFISE	AIIHVLQSKH
	130	140	150	154		
Egyptian fruit bat	PGDFGADAQG	AMGKALELFR	NDIAAKYKEL	GFQG		
Megaleia rufa	AGNFGADAQA	AMKKALELFR	HDMAAKYKEF	GFQG		
Brown-capped capuchin	PGDFGADAQG	AMKKALELFR	NDMAAKYKEL	GFQG		
Australian echidna	SADFGADAQA	AMGKALELFR	NDMATKYKEF	GFQG		

Table D3.2 Amino acid sequences of mammalian myoglobins;. Man langur, Bornean orangutan, Olive baboon and Chimpanzee.

	10	20	30	40	50	60
Man langur	MGLSDGEWQL	VLNVWGKVEA	DIPSHGQEVL	IRLFKGHPET	LEKFDKFKHL	KSEDEMKASE
Bornean orangutan	MGLSDGEWQL	VLNVWGKVEA	DIPSHGQEVL	IRLFKGHPET	LEKFDKFKHL	KSEDEMKASE
Olive baboon	MGLSDGEWQL	VLNVWGKVEA	DIPSHGQEVL	IRLFKGHPET	LEKFDKFKHL	KSEDEMKASE
Chimpanzee	MGLSDGEWQL	VLNVWGKVEA	DIPGHGQEVL	IRLFKGHPET	LEKFDKFKHL	KSEDEMKASE
	70	80	90	100	110	120
Man langur	DLKKHGATVL	TALGGILKKK	GHHEAEIKPL	AQSHATKHKI	PVKYLELISE	SIIQVLQSKH
Bornean orangutan	DLKKHGATVL	TALGGILKKK	GHHEAEIKPL	AQSHATKHKI	PVKYLEFISE	SIIQVLQSKH
Olive baboon	DLKKHGATVL	TALGGILKKK	GHHEAEIKPL	AQSHATKHKI	PVKYLELISE	SIIQVLQSKH
Chimpanzee	DLKKHGATVL	TALGGILKKK	GHHEAEIKPL	AQSHATKHKI	PVKYLEFISE	CIIQVLHSKH
	130	140	150	154		
Man langur	PGDFGADAQG	AMNKALELFR	NDMAAKYKEL	GFQG		
Bornean orangutan	PGDFGADAQG	AMNKALELFR	KDMASNYKEL	GFQG		
Olive baboon	PGDFGADAQG	AMNKALELFR	KDMASNYKEL	GFQG		
Chimpanzee	PGDFGADAQG	AMNKALELFR	KDMASNYKEL	GFQG		

Table D3.3 Amino acid sequences of mammalian myoglobins; Siamang, Agile gibbon, Human and Mountain gorilla.

	10	20	30	40	50	60
Siamang	MGLSDGEWQL	VLNVWGKVEA	DIPSHGQEVL	IRLFKGHPET	LEKFDKFKHL	KSEDEMKASE
Agile gibbon	MGLSDGEWQL	VLNVWGKVEA	DIPSHGQEVL	IRLFKGHPET	LEKFDKFKHL	KSEDEMKASE
Human	MGLSDGEWQL	VLNVWGKVEA	DIPSHGQEVL	IRLFKGHPET	LEKFDKFKHL	KSEDEMKASE
Mountain gorilla	MGLSDGEWQL	VLNVWGKVEA	DISGHGQEVL	IRLFKGHPET	LEKFDKFKHL	KSEDEMKASE
	70	80	90	100	110	120
Siamang	DLKKHGATVL	TALGGILKKK	GHHEAEIKPL	AQSHATKHKI	PVKYLEFISE	CIIQVLQSKH
Agile gibbon	DLKKHGATVL	TALGGILKKK	GHHEAEIKPL	AQSHATKHKI	PVKYLEFISE	CIIQVLQSKH
Human	DLKKHGATVL	TALGGILKKK	GHHEAEIKPL	AQSHATKHKI	PVKYLEFISE	CIIQVLQSKH
Mountain gorilla	DLKKHGATVL	TALGGILKKK	GHHEAEIKPL	AQSHATKHKI	PVKYLEFISE	CIIQVLQSKH
	130	140	150	154		
Siamang	PGDFGADAQG	AMNKALELFR	KDMASNYKEL	GFQG		
Agile gibbon	PGDFGADAQG	AMNKALELFR	KDMASNYKEL	GFQG		
Human	PGDFGADAQG	AMNKALELFR	KDMASNYKEL	GFQG		
Mountain gorilla	PGDFGADAQG	AMNKALELFR	KDMASNYKEL	GFQG		

Where,

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
K	lysine
L	leucine
M	methionine
N	asparagines
P	Proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	Valine
W	tryptophan
Y	tyrosine

APPENDIX E
RESEARCH PUBLICATIONS

RESEARCH PUBLICATIONS

1. Chotchayapong, U., Wiangsamut, K., Chanthai, S., Sattaysai, N., and Tsuchiya, T. (2008) **Thermal stability and denaturation rate of myoglobin from stripped-snake head fish (*Ophiocephalus striatus*).** Poster presentation at the Third Annual Symposium of Protein Society of Thailand, August 28-29, Chulabhorn Research Institute Conference Center, Bangkok, Thailand.
2. Wiangsamut, K., Chotchayapong, A., Chanthai, S., and Sattaysai, N. (2009) **Purification and Characterization of myoglobin from (*Monopterus albus*) by using non-denatured polyacrylamide gel electrophoresis.** Poster presentation at PERCH-CIC congress VI, May 3-6, Jomtein Palm Beach Hotel & Resort Pattaya, Chonburi, Thailand.
3. Wiangsamut, K., Chotchayapong, A., Sattaysai, N., and Chanthai, S. (2009) **Purification and Characterization of myoglobin from (*Monopterus albus*).** Poster presentation at the 4th Annual Symposium of Protein Society of Thailand, August 26-28, Chulabhorn research institute conference center, Bangkok, Thailand.

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