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Isolation and Characterization of *Chelonia mydas* Myoglobin

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Green sea turtle – *Chelonia mydas* is a lung respiration animal that is able to dive and stay uder sea water for hours without needing to surface for oxygen. As oxygen supply in muscles is assured by myoglobin, we propose to study some characteristic of this muscle protein in green sea turtles. To achieve this objective, pure green sea turtle myoglobin has to be made available. Therefore, our first task is to purify this muscle protein from green sea turtles. Skeletal muscles from 3 green sea turtle hatchlings were studied microscopically and biochemically. Microscopy observation showed a general structure of striated muscle. Biochemical studies revealed that green sea turtle myoglobin could be more purely isolated to a certain degree by Sephadex G-75 gel filtration and purified by immunoaffinity gel chromatography rather than direct purification by DEAE-Sepharose ion exchange chromatography technique. SDS-PAGE analysis showed that green sea turtle myoglobin ran together with horse myoglobin as 17 kDa molecular weight proteins.

Key words: green sea turtle, myoglobin, molecular weight, isolation, characterization

INTRODUCTION

It is well known that the brain and the heart are the most aerobic organs in the body, and, therefore, both consume a great portion of respiratory oxygen (Halliwell & Gutteridge 2007; Cossins & Berenbrink 2008). Consequently, any disturbance of oxygen flow to these organs will produce deleterious effects. Indeed, nowadays, cardiovascular and cerebrovascular disorders are considered as two of the most important causes of morbidity and mortality (Somero & Suarez 2005; Kiang & Tsen 2006). It seems that aerobic organisms, who take oxygen directly from the atmosphere (Somero & Suarez 2005), cannot tolerate any anaerobic condition for more than several minutes (Kitagawa *et al*. 2008). Otherwise, it will undergo brain or heart anoxia which leads to brain or heart damage (Somero & Suarez 2005; Kiang & Tsen 2006). Even if organ hypoxia is not so severe, the organism will undergo a certain degree of function limitation (Kitagawa *et al.* 2008). In this case, we will find reduced heart function and limited physical activity. However, this is not the case for some marine terrestrial animals. Whales, dolphins and, especially, sea turtles can dive and remain active under water for a relatively long period of time (Bickler & Buck 2007; Kitagawa *et al.* 2008; Vaquer-Sunyer & Duarte 2008; Fossette *et al*. 2010). Even green sea turtles (*Chelonia mydas*) can remain active for several hours under surface of the sea (Bickler & Buck 2007; Vaquer-Sunyer & Duarte 2008; Puspitaningrum *et al*. 2011). Nevertheless, all of these animals respire with lungs (Butler 2006). This means that they cannot extract soluble oxygen directly from seawater (Price *et al*. 2006). One important question arises; how do such animals, especially sea turtles, manage the oxygen taken previously from the atmosphere? It should be very important to study the biological characteristic of myoglobin, a very important oxygen binding molecule that is found in muscles (an organ which supports physical activities) (Halliwell & Gutteridge 2007; Wittenberg 2009; Fossette *et al.* 2010). For this purpose, we must have pure sea turtle myoglobin.

MATERIALS AND METHODS

All of the following protocols, concerning green turtle (*Chelonia mydas*) egg collection, growth condition, hatching method, and the method of euthanizing, has been reviewed and agreed by an independent Ethical Code Committee of the Department of Health - Republic of Indonesia LB.03.02/KE/1479/2008. To obtain green sea turtle hatchlings, their eggs were collected immediately after they were laid down in Pangumbahan beach, south of Sukabumi, West Java, one of several protected natural reserves for green turtles in Indonesia. All of the eggs

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were transported to a laboratory at the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Jakarta - UNJ. The eggs were incubated for 45 days in room temperature 27-31 °C (Stapleton & Eckert 2008; Norton 2005). Green turtle hatchlings were sacrificed and their pectoral muscles were dissected for further studies. Some of the specimens were treated for histological observations and the rest of the samples were used for biochemical studying.

Histological Preparation and Analysis. The muscle specimen was fixed in formaldehyde, followed by a series of treatments that include dehydrating the tissue specimens, embedding them in paraffin blocks, slicing them into thin slices, and mounting tissues on microscopic slides. This was followed by a series of treaments for dehydrating the thin sections and eventually staining them with hematoxylin and eosin solution (Jensh & Fawcett 2002). The muscle sections were studied under a light microscope.

Biochemical Analysis. Muscle specimens were homogenized with a pestle using a Potter-Elvejehm apparatus. The homogenate was refrigerated-centrifuged, and the supernatant was collected as a crude sample (modification of Maeda and Fitch technique) (Maeda & Fitch 1981). In all the experiments, Tris buffer (pH = 8.6), added by anti-protease, was used.

Myoglobin purifications from crude sample of pectoral were obtained in two ways: (i) Myoglobin was filtrated in a Sephadex G-75 column then purified in an immunoaffinity gel chromatography EconoPack 10DG BioRad column. Crude protein samples were poured into the matrix gel affinity that binds anti-human myoglobin antibody (SantaCruz FL-154). Turtle myoglobin protein elution was created from the turtle myoglobin - antibodies anti-human myoglobin complex by flowing urea 6 M; (ii) Myoglobin was purified by DEAE-Sepharose ion exchanged chromatography directly from crude samples. In this technique, proteins were eluted from resin by adding high molarity NaCl solution. The myoglobin containing fractions were collected and dialyzed with a large volume of distilled water at 4 °C. The dialysis was considered complete when the filtrate was free from NaCl. The precipitate within the dialysis bag was dissolved in 5 ml NaOH 0.1 N. The mixture was further analyzed by electrophoresis in polyacrylamide gel using separation buffer containing sodium dodecyl sulfate (SDS). The same gel was also givenprotein markers (GE Health Care MW 14.000-97.000) and pure horse Mb (Sigma no cat. M0630).

RESULTS

Ninety percent of green sea turtle eggs were successfully hatched. Not all of the animals were used in this study. Most of the little green turtle, after reaching a certain age, were returned to the sea by replacing them in the same beach where they were laid as eggs (Doyle *et al*. 2008; Stapleton & Eckert 2008; Norton 2005). Only three green sea turtle hatchlings were used for this study.

Histological Observation. Figure 1 shows the microphotography of a green sea turtle hatchling's skeletal muscle. It can be seen that turtle skeletal muscle has the same general appearance as a normal skeletal muscle (Jensh & Fawcett 2002), as it also has a syncytium structure, an elongated fibrous like cell with multiple nuclei and without any clear border among the long, fiber-like cytoplasm.

Sephadex Gel Filtration. Gel filtrations that were conducted in Sephadex G-75 show the fractionation pattern of tissue proteins consisting of a sharp peak after a void in the volume of crude extract sample (Maeda & Fitch 1981). Therefore, the fraction columns (Figure 2: tube number 13-32) were pooled and used for further analysis.

Immunoaffinity Gel Chomatograhpy. Purification of turtle myoglobin protein from the results of Sephadex gel filtration use immunological reaction. Purification was done by binding myoglobin proteins = to the antibody anti-human myoglobin which was first attached to the matrix gel chromatography EconoPac BioRad 10DG. Furthermore, turtle myoglobin protein elution from the antibody binding was done by adding 6 M Urea, a chaotropic agent. The results obtained are shown in Figure 3 (tube numbers are 31-32).

DEAE-Sepharose Ion Exchange Chromatography. The crude extract tissue protein samples were pooled and further purified in DEAE-Sepharose chromatography

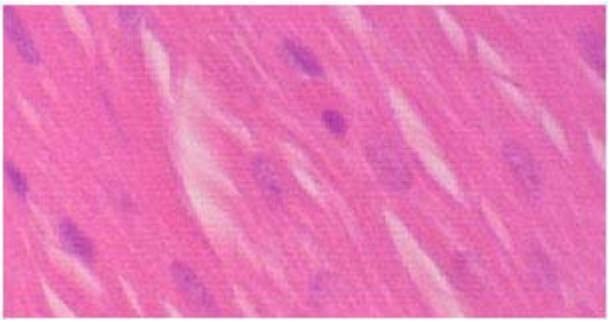


Figure 1. Microphotography of green sea turtle hatch skeletal muscle, hematoxylin-eosin staining.

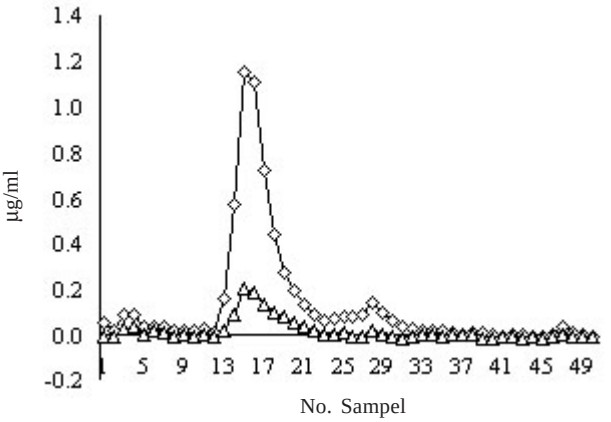


Figure 2. Fractination of green sea turtle hatch myoglobin protein muscle with Sephadex G-75 (no of tube 13-32). —△—: 280nm absorbance values, —◇—: Protein concentration.

(Modification of Maeda & Fitch technique). The pooled fractions were dissolved in NaCl 0.9 g/dl and the myoglobin containing the fractions was eluted from the gel by increasing the NaCl concentration to 9 g/dl. As can be seen in Figure 4, the myoglobin containing fractions form a clear peak after adding the NaCl in higher concentrations.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The fractions that form 2 peaks in DEAE-Sepharose ion exchange chromatography were pooled and analyzed electrophoretically in polyacrylamide gel. The sample was mixed with Tris buffer (pH = 8.6) containing SDS. A solution containing marker proteins was used as molecular weight reference and pure horse myoglobin was used for identification. The results can be seen in Figure 5.

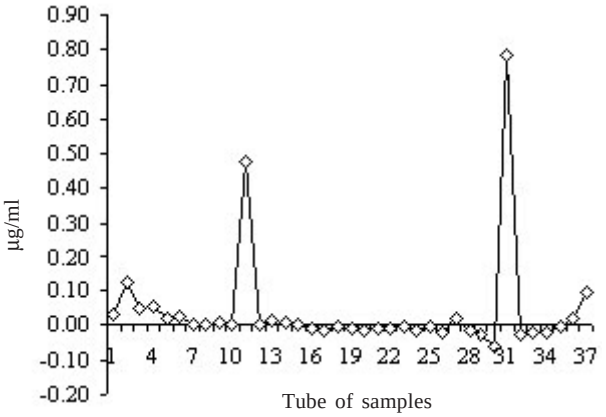
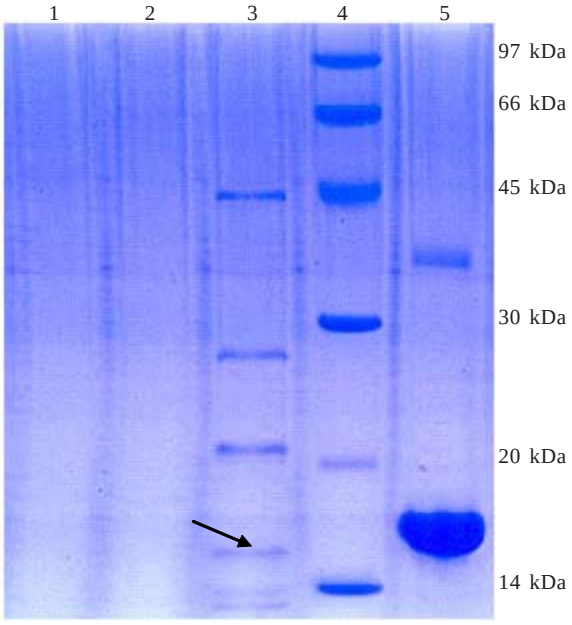


Figure 3. Purification of turtle hacth myoglobin muscle from protein crude sample (tube of 13-32 Figure 2) by using immunoaffinity gel chromatograph EconoPack 10DG - BioRad. Elution of turtle myoglobin fraction of antibody anti-human myoglobin (SantaCruz FL-154) binding on the gel matrix column (no of tube 31-32).



Figures 5. SDS-PAGE electrophoresis of peak 2 DEAE Sepharose (lane 1-3). Lane 6 is marker protein (GE Health Care) and lane 5 is pure horse myoglobin (Sigma M0630 - 17 kDa).

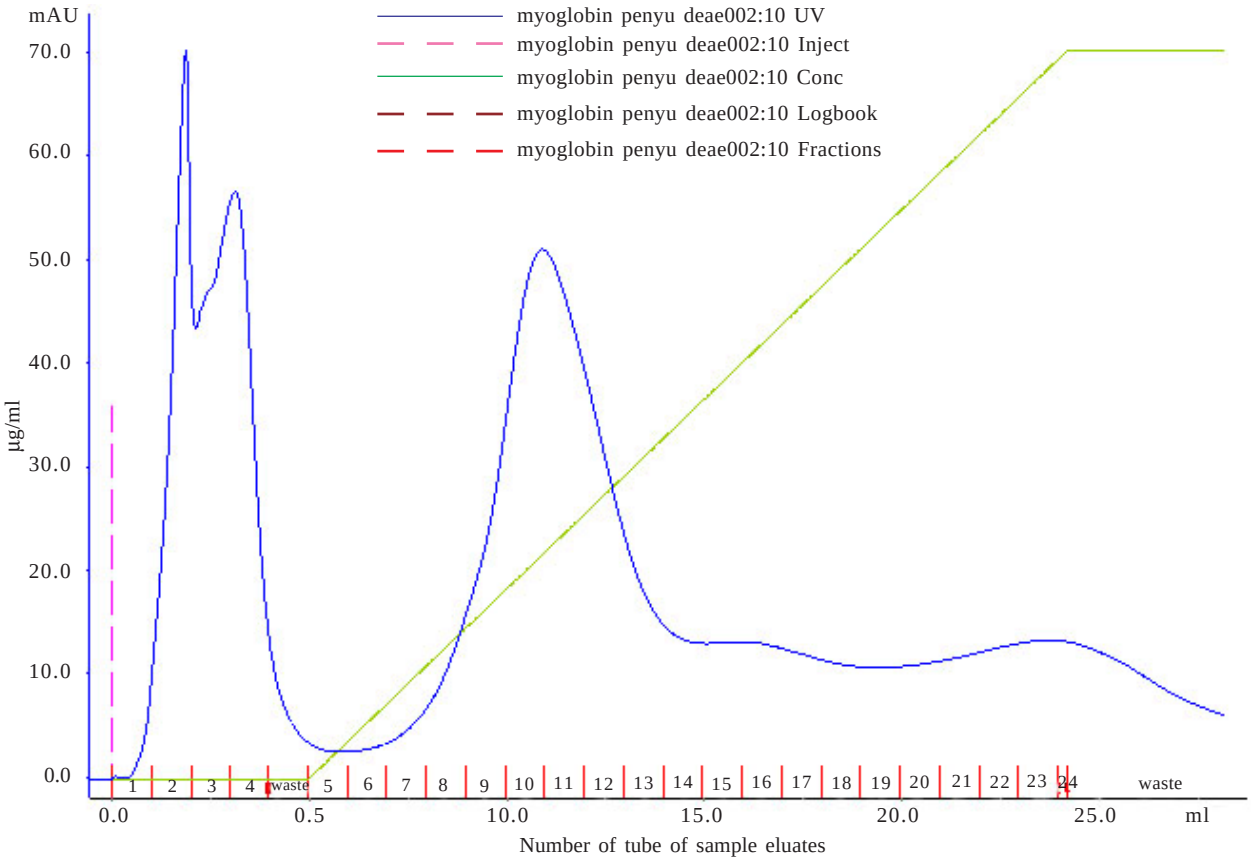


Figure 4. Separation of Mb with DEAE-Sepharose by using AKTA Purifier-Amersham.

Purification of myoglobin from crude protein samples using Sephadex gel filtration technique resulted in good results. This is caused by proteins being cleared from the bond of ammonium sulphate during filtration (Figure 2). Peak protein fractions containing turtle protein myoglobin can be seen in Figure 2. This is evidenced by the results of myoglobin protein purification using immunoaffinity gel chromatography technique (Figure 3). Other proteins contained in the protein fraction have similar molecular weight. Pure turtle myoglobin protein was obtained using affinity gel chromatography technique. The results was obtained from the protein myoglobin elution from antibody anti-human myoglobin binding contained in a gel matrix using urea 6 M.

DISCUSSION

Myoglobin is a binding and oxygen storage protein in tissues (Maeda & Fitch 1981; Halliwell & Gutteridge 2007; Cossins & Berenbrink 2008). When the tissues require a supply of oxygen, myoglobin will release oxygen into the mitochondria. Oxygen is required for energy transduction in mitochondria (Stapleton & Eckert 2008; Wittenberg 2009). Therefore, in any kind of organism, myoglobin has a very important role in the state of the tissue (Bickler & Buck 2007; Vaguer-Sunyer & Duarte 2008; Rayner *et al*. 2009). Turtles are reptiles that breathe with lungs, but most of their life is spent under the sea surface (Hays 2008; Doyle *et al*. 2008; Vaguer-Sunyer & Duarte 2008). Therefore, it was suspected that turtles have a unique myoglobin structure to support them in living under anaerobes state (Bickler & Buck 2007; Kitagawa *et al*. 2008; Vaguer-Sunyer & Duarte 2008; Fossette *et al*. 2010).

The potensial for such evaluation should be enhanched by the availability of data on its myoglobin. To that end we present herein a description of characteristic of green sea turtle myoglobin. These resptiles are noted for their light-colored muscle and deep, prolonged diving. Chelonian may more than 100 m in depth for periods up to 5 hr (Berkson 1966). In this condition, their heart beat has been demonstrated slow one beat per 9 min (Berkson 1967). In the turtles have incapabilities of absorbing oxygen and shows a marked capacity to survive by anaerobiosis (Williams & Brown 1976).

Molecular Weight. Figure 2 show the straight line relationship between Kav and log molecular weight as determined on a sephadex G-75 column. Green sea turtle myoglobin exhibited virtually the same rate as horse myoglobin, emerging just slightly earlier. When the two myoglobins were mixed and applied to the column, no measurable separation was monitored. Molecular weight for the turtle myoglobin, as determined by gel filtration, thus appears to be about 17 kDa or slightly higher.

Myoglobin Green Sea Turtle Protein Homologous. Based on test results of protein purification myoglobin using immunological reaction of antibodies anti-human myoglobin immunoaffinity chromatography on matrix gels, it is known that sea turtle myoglobin molecule structure is homologous to human myoglobin (Figure 3) (Harrera & Lehmann 1971b; Watts *et al*. 1983; Puspitaningrum 2010; Puspitaningrum *et al*. 2010). This is evidenced by the results of turtle myoglobin protein elution from the antibody binding anti-human myoglobin. This situation illustrates that the ability of live turtles in the sea water is not indicated by differences in the molecular structure of myoglobin (Brown *et al*. 1962). Under this condition, it can be concluded that the tolerance of an organism in a state of hypoxia can be created (Butler 2006). However, other characteristics of turtle myoglobin should be further investigated to get a better explanation of how the turtle tissues maintain oxygen homeostasis during long periods of time underwater.

Tolerance to hypoxia in an organism can be developed through the mechanisms of adaptation.An example is the increase of myoglobin content through physical activity or exercise routines (Kanatous *et al.* 2009; Takamura *et al*. 2010). Increased levels of myoglobin in tissues will increase the partial pressure of myoglobin which ultimately increases the binding oxygen affinity in tissues (Kanatous *et al*. 2009; Lunby *et al.* 2009; Takamura *et al*. 2010). These circumstances may explain why people who suddenly experience an acute pathological condition of hypoxia can survive (e.g. cardiac ischemia or stroke) (Somero & Suarez 2005; Cossins & Berenbrink 2008; Kanatous *et al.* 2009).

Turtle myoglobin protein purification from crude protein sample directly using the technique of gel DEAE ion exchange chromatography did not yield better results. Myoglobin protein purification results produced by this method differ from the results obtained by purification using advanced filtration techniques (Figure 3-5). Known results of myoglobin protein purification using DEAE gel technique yield a peak fraction that still contains more than three types of proteins (Figure 5). This means that to obtain pure myoglobin proteins from green sea turtles, more sensitive and specific purification techniques, such as immunoaffinity chromatography gel, are needed.

Figure 4 lane 3 contain post DEAE-Sepharose proteins. A myoglobin protein band is detected in lane 3, at the same level with 17 kDa region in lane 5. On the other hand, in lane 5, pure horse myoglobin migrates exactly at similar distances with protein identified in lane 3 at the 17 kDa level. Thus it can be concluded that we have isolated and purified to a certain degree of purity a group of proteins containing myoglobin from green sea turtle hatchling muscle. Moreover, green sea turtle myoglobin has the same molecular weight as a horse myoglobin (Puspitaningrum *et al.* 2010).

It has been well known that myoglobin is essential for assuring oxygen supply, especially in striated muscle cells such as skeletal muscle and heart muscle (Halliwell & Gutteridge 2007; Wittenberg 2009). Empirically, it has also been well known that more aerobically active animals have muscles that are more dark red, as can be seen in the horse muscle, pigeon breast muscle, shark and tuna muscle (Brown *et al*. 1962; Harrera & Lehmann 1971a; Maeda & Fitch 1981; Bickler & Buck 2007; Vaguer-Sunyer & Duarte 2008). Physiologically, all of them are aerobic animals. For supporting their activities, they need a greater amount of

energy, which is met only by aerobic oxidation (Merx *et al*. 2005; Kitagawa *et al*. 2008; Wittenberg 2009). For reach this objective, the oxygen not only must be able to enter the muscle cell, but also must be dissolved in great amounts. This condition can be achieved only if oxygen is bound chemically and not solely dissolved physically. This condition is exactly the role of the myoglobin (Merx *et al*. 2005; Somero & Suarez 2005; Butler 2006; Foss & Keteyian 2006; Doyle 2008; Wittenberg 2009).

This study showed only the presence and some basic physicochemical character of sea turtle myoglobin. It is not a surprise that the character resembles, in some aspect, horse myoglobin, an animal which is well known for its aerobic activities. Our observation cannot yet describe other properties such as the muscle myoglobin content, its oxygen affinity, and the amino acids sequences homologies. Some of these characteristics, we hope, can be published in following studies.

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REFERENCES

Berkson H. 1966. Physiological adjustmens to prolonged diving in the Pacific Green turtle (*Chelonia mydas* Agassi). *Com Biochem Physiol* 11:81-87.

Berkson H. 1967. Physiological adjustmens to deep diving in Pacific Green turtle (*Chelonia mydas* Agassi). *Com Biochem Physiol* 21:507-524. http://dx.doi.org/10.1016/0010-406X(67)90448-3

Bickler PE, Buck LT. 2007. Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. *Ann Rev Physiol* 69:145-170. http://dx.doi.org/10.1146/annurev.physiol.69.031905.162529

Brown WD, Martinez M, Johnstone M, Olcott HS. 1962. Comparative biochemistry of myoglobins. *J Biol Chem* 244:2149-2158.

Butler PJ. 2006. Aerobic dive limit. what is it and is it always used appropriately? *Comp Biochem Physiol A Mol Integr Physiol* 145:1-6. http://dx.doi.org/10.1016/j.cbpa.2006.06.006

Cossins A, Berenbrink M. 2008. Physiology: Myoglobin's new clothes. *Nature* 454:416-417. http://dx.doi.org/10.1038/454416a

Doyle T, Houghton JO, Súilleabháin P, Hobson V, Marnell F, Davenport J, Hays G. 2008. Leatherback turtles satellite-tagged in European waters Endangered Species. *Research* 4:23-31. http://dx.doi.org/10.3354/esr00076

Foss ML, Keteyian SJ. 2006. *Physiological basis for exercise and sport*. Philadelphia, Pensylvania: Alsevier Saunders. p 79-80;530;1056-607.

Fossette S, Gleiss AC, Myers EY, Garner S, Liebsch N, Whitney NM, Hays GC, Wilson RP, Lutcavage ME. 2010. Behaviour and buoyancy regulation in the deepest-diving reptile: the leatherback turtle. *J Exp Biol* 213:4074-4083. http://dx.doi.org/10.1242/jeb.048207

Halliwell B, Gutteridge JMC. 2007. *Free radicals in Biology and medicine* (4th ed.). Oxford Univ Pr.

Harrera AER, Lehmann H. 1971a. The myoglobin of primates-I. *Hylobates agilis* (gibbon). *Biochem Biophys Acta* 251:482-488.

Harrera AER, Lehmann H. 1971b. Primary structure of human myoglobin. *Nature Biol* 232:149-152.

Hays GC. 2008. Sea turtles: a review of some key recent discoveries and remaining questions. *J Exp Mar Biol Ecol* 356:1-7. http://dx.doi.org/10.1016/j.jembe.2007.12.016

Jensh RP, Fawcett DW. 2002. Bloom & Fawcett's Concise Histology. 2nd ed. London: Arnold Publ. p 110-115.

Kanatous SB, Pradeep PA, Mammen PB, Rosenberg CM, Martin MD, White J, Michael D, Guojin H, Shmuel M, Daniel JG. 2009. Hypoxia reprograms calcium signaling and regulates myoglobin expression. *Am J Physiol Cell Physiol* 296:C393-C402. http://dx.doi.org/10.1152/ajpcell.00428.2008

Kiang JG, Tsen KT. 2006. Biology of hypoxia. *Chinese J Phyisiol* 49:223-233.

Kitagawa H, Yamazaki T, Akiyama T, Nosaka T, Mori H. 2008. Acta Isoflurane attenuates myoglobin release during ischemic and/or reperfusion periods. *Acta Anaesthesiologica Scandinavica* 52:650-657. http://dx.doi.org/10.1111/j.1399-6576.2008.01601.x

Lundby C, Calbet JA, Robach P. 2009. The response of human skeletal muscle tissue to hypoxia. *Cell Mol Life Sci* 66:3615-3623. http://dx.doi.org/10.1007/s00018-009-0146-8

Maeda N, Fitch WM. 1981. Amino acid sequence of a myoglobin isolated from map turtle, *Gmptemys geographic. J Biol Chem* 256:4293-4300.

Merx MW, Godecke A, Flogel U, Schrader. 2005. Oxygen supply and nitric oxide scavenging by myoglobin contribute to exercise endurance cardiac function. *Faseb J* 19:1015-1017.

Norton TM. 2005. Chelonian emergency and critical care. *J Cambridge* 14:106-130.

Price WR, Paladino FV, Strohl KP, Santidrián TP, Klann K, Spotila JR. 2006. Respiration in neonate sea turtles. *Comp Biochem Physiol A Mol Integr Physiol* 146:422-428. http://dx.doi.org/10.1016/j.cbpa.2006.11.034

Puspitaningrum R. 2010. Ekspresi dan karakterisasi molekul mioglobin tukik *Chelonia mydas* sebagai model protein pengikat dan penyimpan oksigen hewan toleran hipoksia [Disertasi]. Jakarta: Universitas Indonesia.

Puspitaningrum R, Wanandi SI, Soegianto RR, Sadikin M. 2011. Turtle hatch *Chelonia mydas* blood gasses analysis in different field temperature. *HAYATI J Biosci* 18:147-157. http://dx.doi.org/10.4308/hjb.18.3.147

Puspitaningrum R, Wanandi SI, Soegianto RR, Sadikin M, Williams DR, Cossins AR. 2010. Myoglobin expression in *Chelonia mydas* brain, heart and liver tissues. *HAYATI J Biosci* 17:110-114. http://dx.doi.org/10.4308/hjb.17.3.110

Rayner BS, Hua S, Sabaretnam T, Witting PK. 2009. Nitric Oxide stimulates myoglobin gene and protein expression in vascular smooth muscle. *Biochem J* 423:169-177. http://dx.doi.org/10.1042/BJ20090716

Somero GN, Suarez RK. 2005. Peter Hochachka: Adventures in Biochemical Adaptation. *Annu Rev Physiol* 67:25-37. http://dx.doi.org/10.1146/annurev.physiol.67.041904.120836

Stapleton SP, Eckert KL. 2008. Community-Based Sea turtle research and conservation in Dominica: a manual of recommend practices. Dominica. WIDECA ST and the Dominica sea turtle conservation organization (DomSeTCO).

Takamura, Masuda K, Hashimoto T, Iwase S, Jue T. 2010. Quantification of myoglobin deoxygenation and intracellular partial pressure of O_2 during muscle contraction during haemoglobin-free medium perfusion. *Exp Physiol* 95:630-640. http://dx.doi.org/10.1113/expphysiol.2009.050344

Vaquer-Sunyer R, Duarte CR. 2008. Thresholds of hypoxia for marine biodiversity. *PNAS* 105:15452-15457. http://dx.doi.org/10.1073/pnas.0803833105

Watts DA, Angelides T, Brown WD. 1983. The primary structure of myoglobin from pacific green sea turtle (Chelonia mydas caranigra). *BBA* 742:310-317.

Wiliams JD, Brown DW. 1976. Characterization of myoglobins from atlantic and pacific green sea turtles. *Comp Biochem Physiol* 54B:253-259.

Wittenberg BA. 2009. Both hypoxia and work are required to enhance expression in myoglobin in skeletal muscle. *AJP Cell Physiol* 296:C391-C392.