Comparison of Hemoglobins from Various Subjects Living in Hypoxia

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The aim of this research was to obtain the different characteristics of haemoglobin molecules in subjects under hypoxic condition, namely eel, catfish, suckermouth fish, green sea turtle using an electrophoresis technique. We used human umbilical cord blood and thalassemia patient blood, as well as a normal adult-human blood as controls. The proteins obtained after electrophoresis process were stained with two different colouring techniques, each based on different principles. Both staining techniques gave practically identical results. Subject that live in hypoxic condition has a different haemoglobin in comparison to the one found in adult human live in normal oxygen condition (normoxia). These hypoxia-adapted or -needed hemoglobin migrate slower than adult human hemoglobin from normoxia. This observation suggests that hemoglobin which is needed to live in hypoxic condition or environment is a different molecule. Whether this hemoglobin from hypoxic condition has a higher affinity to oxygen is not yet known. Further study is needed to clarify this issue.

Keywords: *Monopterus albus, Clarias bathracus, Hyposarcus pardalis, Chelonia mydas,* human umbilical cord, β Thalassemia Patient and Human adult, haemoglobin protein content

INTRODUCTION

Each species has a specific habitat. The habitat determines their physiological and genetic characteristics. Some animals have a low oxygen environment as their habitats (Wheaton & Navdeep 2011). Others have to live in a relatively low oxygen environment in a certain stadium of their development or in a certain pathological condition, for instance in the embryonic state or in a congenital anemic condition (Akinsheye *et al*. 2011). All of these conditions compel the related organism to adapt to the specific habitat in order to survive (Roshan *et al*. 2011).

As oxygen is very essential for animals or even for simpler multi-cellular organisms, low oxygen environment necessitates them to dispose a more efficient or more powerful molecule for extracting the oxygen in such a low amounts (Schuyler *et al.* 2012). It is well known that such a molecule is hemoglobin (Leichtle *et al*. 2011). Hence, it is reasonable to think that this oxygen-binding molecule might have a different characteristic in such animals or condition.

This suggestion is based on the hemoglobin characteristics found in red blood cells that serve to bind oxygen in the blood of vertebrates (Kanias & Jason 2010; Richards 2011). Hemoglobin has to be able to bind O_2 in a relatively low concentration (Cabrales *et al.* 2011). This is possible only if the hemoglobin has a higher affinity to O_2 than hemoglobin in normal condition (Rao *et al*. 2010). As the hem group remains the same, any difference should be explored in globin protein itself (Noosud *et al.* 2010). If this is the case, the difference in the globin protein could be reflected in the physicochemical properties of the hemoglobin. Usually, a difference between 2 proteins, even only in 1 amino acid residue, could be revealed by electrophoretic analysis (Signore *et al.* 2012). The objective of this experiment was to analyze the difference of electrophoretic pattern of hemoglobins from several subjects living in a relatively hypoxic condition and also from fetus and thalassemic patient (Wajcman & Kamran 2011).

Subjects that are adapted to live in low-oxygen environments were represented by an eel (*Monopterus albus*), a catfish (*Clarias batrachus*), a suckermouth fish (*Hyposarcus pardalis*), a green turtle (*Chelonia mydas*) and a new-born baby's umbilical cord. The blood sample of thalassemic patient was used as an oxygen-binding disability example.

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MATERIALS AND METHODS

Chelonia mydas is an endangered animal and was obtained from Pangumbahan Beach Sukabumi, West Java Island. This animal has CITES certificate from Ministry of Forestry Republic of Indonesia: SK/136/IV-SET/2008. Our courtesy to Prof. dr. Mohamad Sadikin, D.Sc., for the permission to use collection of human blood samples stored as a control in the laboratory of Biochemistry and Molecular Biology, Faculty of Medicine, University of Indonesia. Eels, catfish and suckermouth fish samples, all were kind gift from Karunia Nutzir Mantolini from State University of Jakarta.

Preparation of Hemolysate. Blood was washed and suspended in 0.9% NaCl in order to eliminate all plasma protein. One volume of blood was well mixed with 1 volume of 0.9% NaCl and the suspension was centrifuged 300 rpm for ten minutes. The supernatant was discarded. The process was repeated three times until the supernatant was free from plasma protein and the hemoglobin leaked from the destructed erythrocyte. The washed blood cells are called pack cells.

Hemoglobin Isolation. Pack cells were mixed with distilled water and CCl_4 in 1:2:1 proportion (pack cells:distilled water:CCl_4). The mixture was well mixed and centrifugated at 300 rpm for ten minutes.

Hemoglobin Analysis by Cellulose Acetate Membrane Electrophoresis. Supre-Heme® Helena Buffer (Cat. No. 5802) which comprised of Tris-EDTA and borate acid was diluted in 980 ml of distilled water. Titan® disc III acetate cellulose (Cat. No. 3021) was soaked in the Supre-Heme® Helena buffer for 10 minutes. Ten ml of that buffer was poured into each electrode compartment of the electrophoresis apparatus. Later on, electrophoresis plate (Titan® disc III acetate cellulose (Cat. No. 3021) was placed into the electrode compartment of the electrophoresis apparatus, then it was blotted by placing the plate between two pieces of filter papers. Five microliters of hemolysate were picked up with an applicator and then placed on the electrophoretic plate. After five seconds, the plate containing the lysate was immediately place in the electrophoresis apparatus with the acetate cellulose side faced to the bottom. Electrophoresis was run at 350 Volts and set for 25 minutes. The apparatus stopped automatically after this period. The electrophoresis process was followed with Ponceau S (Cat. No. 5526), and benzidine [Hartman Leddone Company, Philadelphia] staining for each disc with Titan® III Cellulose Acetate membrane. The results were then compared and analyzed.

Ponceau Staining. After the electrophoretic separation, Titan® disc III acetate cellulose (Cat. No. 3021) was dipped in the Ponceau S for five minutes. The plate was stained red. To eliminate all non specific red color, the plate was washed three times in 5% acetate acid solution, two minutes each time. The non specific red color on Titan III-H Plate was gradually diminished and only red protein bands were left. Acetate cellulose membrane was dried in an oven at 56 °C for ten minutes afterward.

Benzidine Staining. The separation plate was placed in a jar for Benzidine staining. The plate was layered with Benzidine solution containing 200 ml distilled water, 0.4 g Benzidine, 1.0 ml glacial acetic acid, and 0.4 ml of 30% H_2O_2. Peroxidase activities of hemoglobin were revealed by the formation of blue bands or spots within the first 20 minutes, then the color of the bands gradually changed to green-brown. After 20 minutes of staining, Titan III-H Plate was washed in a solution of methanol [Merck], and distilled water (1:1).

RESULTS

The electrophoretic separation of eel, catfish, green turtle, suckermouth fish, umbilical cord, thalassemic patient and normal adult human blood showed different hemoglobin migration patterns. Both staining, Ponceau and Benzidine, showed identical migration pattern and distances of hemoglobin bands (Figure 1A & B).

The image of stained proteins or hemoglobins were then analyzed and edited by using Adobe Photoshop CS4. The editing process was aimed to change the background to dark and hemoglobin migration bands to bright colors. Figures will automatically change to black and green or purple (Figure 2 & 3). The migration distance of each hemoglobin band after Ponceau staining is shown in Figure 2, while hemoglobin electrophoresis results with Benzidine staining is shown in Figure 3.

The migration distance of each hemoglobin band, after Ponceau or benzidine staining was measured from the starting point of application to the ultimate frontier of each hemoglobin band. The results can be seen in Table 1.

From Figures 1A and B, Figure 2 and 3, we can see different hemoglobin band migrations. There were three similar bands showed both by thalassemic blood and normal adult human blood. The direction of migration was also very interesting to be seen. While all hemoglobin in this study migrated to the cathode (positive pole), the sucker mouth fish hemoglobin migrated slightly to the contrary direction, the anode (negative pole).
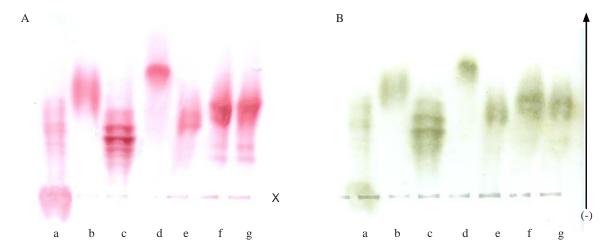
Figure 1. Result of electrophoresis of hemoglobin samples and control application on Titan III cellulose acetate membrane. A. Ponceau straining, B. Benzidine straining, X: Hemolysate application, a: Hb of sucker mouth fish *Hyposarcus pardalis,* b: Hb of catfish *Clarias Bathracus,* c: Hb of eel *Monopterus albus,* d: Hb of *Chelonia mydas,* e: Hb of human umbilical cord blood, f: Hb of β thalassemia patient, g: Hb of human adult.
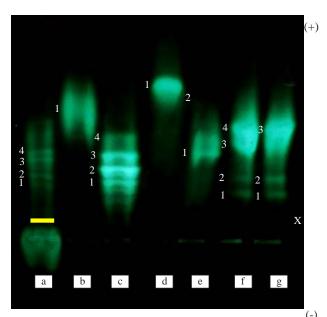

Figure 2. Hemoglobin electrophoretic pattern after Ponceau staining and editing process using Adobe Photoshop CS4. Numbers next to haemoglobin migration bands are markers for discussion purposes. X: Hemolisate application dot, a: Hb of suckermouth fish *Hyposarcus pardalis*, b: Hb of catfish *Clarias Bathracus,* c: Hb of eel *Monopterus albus,* d: Hb of *Chelonia mydas*, e: Hb of human umbilical cord blood, f: Hb of beta thalassemia patient, g: Hb of human adult.
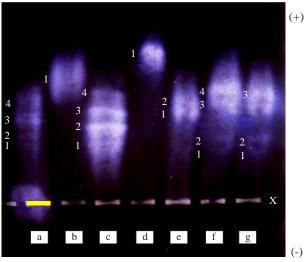

Figure 3. Hemoglobin electrophoresis pattern after benzidine staining and editing process using Adobe Photoshop CS4. Numbers next to hemoglobin migration bands are markers for discussion purposes. X: Hemolisate application dot, a: Hb of suckermouth fish *Hyposarcus pardalis,* b: Hb of catfish *Clarias Bathracus,* c: Hb of eel *Monopterus albus,* d: Hb of *Chelonia mydas,* e: Hb of human umbilical cord blood, g: Hb of beta thalassemia patient, f: Hb of human adult.

DISCUSSION

In this study, we try to understand if the difference of oxygen binding capacities from various species or individuals who are habituated to live in a relatively low oxygen pressure can be reflected by the electrophoretic migration pattern of their hemoglobin (Leichtle *et al.* 2011). We chose the hemoglobin because it is the only protein which bind directly and immediately the environmental oxygen, in air as well as soluble in water (Criner *et al.* 2010). Based on an assumption that various hemoglobin has a similar heme moiety, it can be predicted that oxygen binding capacities difference of hemoglobin must rely on the difference of its protein component (i.e. the globin) (Roshan *et al.* 2011). If this is the case, then the difference between the globin parts must be based on the amino acid sequences, which, in their turn could be reflected in the molecular weight and in the pI (isoelectric pH) (Tan *et al.* 2009). The most

Table 1. Samples' haemoglobin bands from electrophoresis application dots and thickness

Haemoglobin samples	Band	Migration distance of hemoglobin from electrophoresis hemoglobin application dots		Thickness of hemoglobin band (pixel)	
		Ponceau staining	Benzidine staining	Ponceau staining	Benzidine staining
Suckermouth fish *Hyposarcus pardalis*	1	75	80	2	5
	2	81	93	5	4
	3	102	114	5	11
	4	112	144	6	18
Catfish *Clarias bathracus*	1	160	170	19	17
Eel *Monopterus albus*	1	73	81	7	6
	2	85	103	13	12
	3	109	125	6	10
	4	137	154	3	7
Green sea turtle *Chelonia mydas*	1	200	206	17	16
Human umbilical cord	1	113	126	7	9
	2	131	140	7	12
Beta thalassemia patient	1	58	69	8	6
	2	79	87	6	7
	3	127	124	6	19
	4	147	146	9	9
Human adult	1	57	70	9	6
	2	78	86	6	7
	3	146	149	15	9

convenient, easiest but reliable method to study physicochemical properties is electrophoresis technique. Any difference in migration pattern of 2 molecules must be caused by the two physicochemical characteristic, molecular weight and pI (Sadikin *et al.* 2012). This, of course, is directly related to the total number of amino acids and also to the number of charged amino acids (Puspitaningrum *et al.* 2010). Based on this assumption, we used 3 spesies of fish which live in the muddy bead of river or pond (Richards 2011), like eel, catfish and sucker mouth fish (Olayemi 2012). We compared with the hemoglobin from sea turtle, umbilical cord blood and also thalassemic patient blood. We deliberately compared the mud fish hemoglobin with these lung-respiration individual hemoglobin, for 2 reason. Sea turtle, newborn and patient, all are terrestrial organisms that breathe the oxygen from the atmosphere, but sea turtle spend most of its time under sea water (Schuyer *et al.* 2012). Cord blood is fetal blood when its stayed in the womb, which means a hypoxic or event an anoxic condition (there is no free oxygen in the amniotic sac). The thalassemic patient undergoes a relatively hypoxic condition, because of the anemic condition (Bain 2010).

In this experiment, CCl_4 was used as a hemolytic agent. The lipid membrane of erythrocytes was dissolved by this organic solvent and the hemoglobin was liberated (Capote *et al.* 2011). However, this red protein itself is a water-soluble molecule and the hemoglobin samples were taken from the upper part, the water layer.

As a protein, hemoglobin is a charged molecule when dissolved in a buffer whose pH is different from the protein pI (Qu *et al.* 2011). In this experiment, we used a buffer solution with alkaline pH (8.6) as an electrophoretic buffer. Because most protein have pI around 5-6, they are charged negatively and will migrate toward the anode. In our experiment, all hemoglobin migrated toward positive pole (anode) (Unnerstales & Maler 2012). Nevertheless, there was one type of hemoglobin which migrated slightly toward the cathode. We can conclude that the sucker mouth fish hemoglobin has a pI which, in contrary to most protein, is relatively alkaline. This means that the globin, protein part of hemoglobin, in this animal is very different from the other hemoglobin. Proteins in an electrophoretic analysis can be revealed by various stainings, like Ponceau staining, amido black staining or Coomassie crystal blue staining. In this study, we used the Ponceau staining because this technique is fast and simple, yet still specific in coloring any protein. However, because we work with cell lysate (in this case hemolysate), we suppose that there could be other proteins beside hemoglobin in the hemolysate. That is why we stained also the electrophoretic plate with benzidine. This technique is not based on protein property of hemoglobin, but on the peroxidase activity of hemoglobin. This protein can also act as a peroxidase, by catalyzing the H_2O_2 breakdown using benzidine as an electron donor. Consequently the benzidine is oxidized which give rise to dark blue color. In this study, we found that both staining techniques give practically the same

results. Both techniques give the same number of bands in each sample and same pattern of migration. We can conclude that practically hemoglobin is the major protein in erythrocyte.

Normally, adult hemoglobin in human (HbA) has the fastest migration toward the positive pole in the electrophretic field (Gyasi *et al.* 2012). In this experiment, this is represented by band 3 of lane g, band 4 of lane f of thalassemic blood, and band 2 of lane e of human umbilical cord blood. All these bands migrate in a relatively same distances, in Ponceau staining as well as in benzidine staining. Other bands in umbilical cord blood and in thalassemic blood that migrate relatively slower can be considered as another type of Hb. In umbilical cord blood it will be fetal hemoglobin (HbF). In thalassemic patient, often as compensation mechanism in chronic anemia, blood might contain other type of hemoglobins, among them is HbF. Though it is not yet possible to determine definitely the type other hemoglobin in umbilical cord blood and in thalassemic blood, at least it can be said that the other type of Hb is a compensated Hb. It is well known that HbF has a higher affinity to O_2. The compensated Hb in thalassemic blood might be HbF, HbA_2 or even pathologic Hb (Jones *et al.* 2011). Whether the compensated hemoglobin (other than HbF) have a higher affinities to O_2 is not yet known, though logically it should be the case.

If higher oxygen affinity hemoglobin has a slower migration, than it can be well seen in sucker mouth fish. This fish lives in muddy bead of river, a condition with low oxygen content. The sucker mouth fish hemoglobin is dominated by a type of hemoglobin which relatively immobile or even slightly move to negative pole (cathode). We can suppose that this hemoglobin has a pI near the buffer pH (8.2) or even slightly higher. This, of course has a molecular base in amino acid sequences. The other hemoglobin, though migrate to anodic pole, are still lower than HbA of human blood. The phenomena are also observable in eel (lane c), other fish which lives also in mud. However, catfish, another mud living aquatic animal, has hemoglobin with a migration similar to HbA (lane b). The same phenomenon is observed also in green sea turtle (lane d). Sea turtle breathe with lungs and takes oxygen directly from the air, but catfish uses gills for extracting oxygen from water (Puspitaningrum *et al.* 2011). It is not yet clear, why catfish has a hemoglobin whose electrophoretic pattern similar to HbA.

This electrophoretic observation suggests that hemoglobin needed for living in low oxygen content condition or environment is a different molecule. Whether the hemoglobin has a higher affinity to oxygen is not yet known. Further study is needed to clarify this issue.

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