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Comparison of Hemoglobins from Various Subjects Living in Hypoxia

RINI PUSPITANINGRUM[1]*, KURNIA NUZTIR MANTOLINI[1], RUSDI[1], MOHAMAD SADIKIN[2]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, State University of Jakarta, Jalan Pemuda No. 10 Rawamangun, Jakarta 13220, Indonesia
[2]Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Indonesia, Jalan Salemba 6, Jakarta 10430, Indonesia

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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.

*Corresponding author. Phone/Fax: +62-21-4894909 ext. 21, E-mail: rini_puspitaningrum@yahoo.com

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 ^oC 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).