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Isolated Diaphorase From Bovine Erythrocyte Cannot Reduce Oxidized Cytoglobin (Metcygb)

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Abstract

Background: Cytoglobin (Cygb) is a relatively newly identified globin protein that acts as an oxygen transporter in tissues like hemoglobin (Hb) in erythrocytes and myoglobin (Mb) in muscles. The natural oxidation of the Fe^{2+} ion in its heme group into metglobin (globin-Fe^{3+}) made the loses of oxygen binding functions. It is known metHb and metMb can be reduced enzymatically using diaphorase or cyb5r3. However, metCygb reductase had not been previously identified. This study aims to analyze the reducing activity of bovine diaphorase on metCygb.

Methods: Diaphorase was isolated from bovine erythrocyte and purified using gel filtration and cationic-exchanger chromatography. Its purity was verified by SDS-PAGE and western blot (WB). The metCygb was obtained from Cygb oxidation with potassium ferrocyanide and its reducing activity was determined by spectroscopy.

Results: The diaphorase (MW=30.09 kDa) was purified 10.77-fold from crude enzyme with specific activity against metHb 8.479 U/mg. The purity was confirmed by WB using primary antibody anti-cyb5r3. The purified enzyme reduced metCygb at 0.785 µgmin^{-1}, which was 13.7 times less than the Vmax of metHb.

Conclusions: In conclusion, the purified diaphorase from bovine erythrocytes did not significantly reduce metCygb rather than metHb, a natural substrate in cells.

Keywords: Bovine Erythrocyte, Cytochrome B5 Reductase, Diaphorase, Metcytoglobin, Reduction.

Introduction

Reactive oxygen species (ROS) are natural substances that are occur during redox reactions in cells. Certain conditions (e.g., hypoxia) increase the oxidative environment and very likely to oxidize intra and extracellular proteins, especially redox-sensitive proteins such as globin proteins (1, 2). In such a case, ROS levels would be greater than normal, and the oxidation could reach the inner parts of the protein (3, 4). Oxidation of ferrous ions to ferric ions in globin proteins can be fatal for the body because it results in loss of globin function to bind oxygen (5). It is known that 1.5-3% of the hemoglobin (Hb) or myoglobin (Mb) containing ferrous ions will be oxidized spontaneously due to the structural rearrangement to become a ferric ion (6). The oxidized Hb and Mb are called metHb ($HbFe^{3+}$) and metMb ($MbFe^{3+}$). A reduction process is needed to return ferric ions to ferrous ions. The reduction process can take place non-enzymatically or enzymatically (7). Reductases reduce proper substrates using NADH or NADPH as coenzymes (7).

Diaphorase, an enzyme that reduces methemoglobin (Hb-Fe^{3+}) to hemoglobin with reduced Fe (Fe^{2+}), was firstly discovered around 1940. Some research has isolated an enzyme from human erythrocytes that reduces methemoglobin (8–11). In the 1970s, Hagler et

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al. discovered cytochrome b5 reductase, which can reduce metmyoglobin, from bovine heart (12). Several studies reported that diaphorase was the same enzyme as cytochrome b5 reductase (6, 11). NADH will reduce ferric ions in cytochrome b5 (CYPb5-Fe^{3+}) into CYPb5-Fe^{2+}. One molecule of CYPb5-Fe^{2+} can bind to a metHb or metMb subunit through their lysine or arginine residues. This bond causes the transfer of electrons from CYPb5-Fe^{2+} to $HbFe^{3+}$ or $MbFe^{3+}$ to form products CYPb5-Fe^{3+} and $HbFe^{2+}$ or $MbFe^{2+}$ (7, 13).

Cytoglobin is a globin protein first discovered by Kawada et al. (2001) in hepatic stellate cells. It was initially called stellate cell activation-associated protein (STAP). One year later, STAP was cloned and identified as cytoglobin (Cygb) and later found in various organ tissues. It acts as an oxygen supplier in tissues (14). Regarding to the Cygb structure, which resembles Hb and Mb, and considering that iron of both Hb and Mb can be oxidized to metHb and metMb, we asked whether Cygb could also be oxidized to form metCygb, and reduced back to Cygb. Until now, the existence of metCygb reductase was uncertain. Sahara et al. explored the presence of metCygb reductase in bovine liver and showed that the putative enzyme metCygb reductase reduced metCygb than at a greater rate than commercial diaphorase from *Clostridium kluyveri* (15). Because the diaphorase originated from bacteria, we hypothesized that the enzyme would not efficiently reduce animal hemoglobin. Therefore, we decided to purify the diaphorase directly from bovine erythrocytes and observe its ability to reduce metCygb. The purified diaphorase was confirmed using SDS-PAGE, western blot, and reducing activity to metHb. Furthermore, the activity profile of purified diaphorase to reduce metCygb was determined using spectroscopy to determine whether metCygb was also reduced by the same enzyme system as metHb and metMb.

Materials and Methods

Materials included phosphate-buffered saline/PBS (Sigma-Aldrich, USA), ethanol (Merck), chloroform (Merck), NaCl (Merck), acetic acid (Merck), ammonium sulfate (Merck), Sephadex G75 (Sigma-Aldrich), CM Sepharose Agarose (Creative Biomart), Precision plus all blue standard (Bio-Rad), SDS-PAGE reagent kit (Bio-Rad), goat anti-human CYB5R3 (Santa Cruz), rabbit anti-goat IgG conjugated HRP (Abcam), human CYB5R3 recombinant (Abcam), bovine serum albumin/BSA (Santa Cruz), nitrocellulose membrane (Bio-Rad), Tween-20 (Merck), skim milk (NZMP), and Betazoid DAB (Biocare Medical).

Bovine erythrocytes from a slaughterhouse served as the diaphorase source. All experiments were conducted in the laboratory of the Center of Hypoxia and Oxidative Stress Studies, Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia. The ethical requirements were approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (No. ND-510/UN2.F1/ETIK/PPM.00.02/2020).

Isolation of NADH-diaphorase from bovine erythrocyte

The enzyme was extracted according to Huenneken et al. method with some modification (9). First, fresh bovine blood was collected with anticoagulant EDTA. Next, erythrocytes were separated by centrifugation (4℃, 2300 x g), and washed three times with saline. Then, erythrocytes were suspended to their initial volume with saline. To make hemolysate, the suspension was freeze-thawed. Hemoglobin was separated from hemolysate with a cold ethanol: chloroform: distilled water= 0.1v:0.25v:0.15v). This procedure reduces hemoglobin solubility and denatures its homotetrameric structure (16). The mixture was centrifugated and the supernatant was separated from the hemoglobin and dialyzed in distilled water for 4 hr at 40℃. The supernatant's pH was made

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to 6.5 with acetic acid 1 N. The formed precipitate was removed, and the supernatant was neutralized with NaOH 1 N. The supernatant was fractionated with ammonium sulphate and the precipitate of 90% saturated ammonium sulphate was separated (17-20). The precipitate was resuspended in 0.1 M PBS pH 6.8 and dialyzed to remove the ammonium sulphate. This dialysate was stored for further purification.

Purification using gel filtration chromatography

The dialysate was purified by gel filtration column chromatography using resin Sephadex G75. The 1 g resin was swollen in distilled water for 30 min to make a slurry. Then, the slurry was poured into a vertical column and washed with three times the column volume of distilled water. Next, the resin was washed with 0.1 M PBS pH 6.8 and soaked in 12-15 mL of PBS. After that, the resin was equilibrated with 0.1 M PBS pH 6.8 until the 280 nm absorption was stable near zero. The sample was applied to the resin surface (5% of the resin volume), eluted using the same buffer, and 1.5 mL eluates were collected. The elution process was stopped when the 280 nm absorption was stable near zero. The fractions that demonstrated Soret peaks were collected for further purification.

Purification using cation exchanger chromatography

The Soret peak fractions from gel filtration chromatography were further purified on a cation exchange column using CM-Sepharose. The resin was previously swollen in 20% ethanol. The slurry was poured into a vertical column and washed with three times the column volume of 0.01 M PBS pH 6.8 as equilibration buffer. Then, the resin was equilibrated until the 280 nm absorption was stable near zero. The sample was applied to the resin surface (5% of the resin volume), eluted using 0.05 M PBS pH 6.8, and 1.5 mL eluates were collected. This first elution process was stopped when the 280 nm absorption was stable near zero. The buffer was replaced with 0.05 M PBS pH 6.8 containing 0.25 M NaCl for the second elution. The elution was stopped when the 280 nm absorption was stable near zero. The fractions forming peaks were collected for further analysis.

Enzyme specific activity test against bovine methemoglobin

Before carrying out a specific activity test, it is necessary to determine the maximum wavelength of the substrate (metHb) and reaction products (deoxyHb). Met-Hb was prepared by adding 1 mM $K_3Fe(CN)_6$ solution into a 5 µM bovine Hb solution (ratio 1:50). Into the met-Hb solution, a Stokes solution was added (ratio 1:10) to prepare deoxyHb. The maximum wavelength was determined with spectrophotometer scanning from 500-700 nm. The maximum wavelength of met-Hb and deoxy-Hb respectively were 630 and 540 nm. The enzyme activity was assayed following the procedure described in Table 1. The substrate buffer contained 0.2 M PBS pH 7.5 (0.60 mL), met-Hb substrate (0.10 mL), 10 mM NADH (0.10 mL), and 0.20 mL distilled water.

Table 1. Enzyme activity test reagents.

Reagent	Control (c)	Sample (s)
Substrate buffer	1 mL	1 mL
Incubated at 37^0C, 5 minutes		
Enzyme	-	100 µL
Distilled water	100 µL	-
Incubated at room temperature, 10 minutes		
Stop solution (HCl 0,1 N)	2 mL	2 mL
Read the absorption at 540 nm		
Total volume	3.1 mL	3.1 mL

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The enzyme-specific activity was determined by following equation.

$$\text{Enzyme activity (U/mL)} = (\text{As} - \text{Ac})\ \text{x}\ \left(\frac{\text{Vtotal}}{\text{Venzyme}}\right)\text{x}\frac{1}{\text{t}}\text{x}\ (\text{Cs x Vs})$$

$$\text{Specific activity (U/mg)} = \frac{\text{Enzyme activity}}{\text{Protein concentration}}$$

Where As and Ac are the absorbances of sample and control, V is volume, t is time, and C is concentration.

SDS-PAGE and Western Blotting (WB)

SDS-PAGE was performed under denatured sample conditions with a β-mercaptoethanol sample buffer. The 5% stacking gel and 12% running gel were used in this method. Samples were electrophoresed for 60 minutes at 150 V and stained with Coomassie Brilliant Blue. The WB analysis was performed by a semi-dry electrotransfer from gel polyacrylamide onto the nitrocellulose membrane. The electrotransfer was performed for 30 minutes at 25 V and 400 mA (constant current). After the transfer, the nitrocellulose membrane was soaked in Ponceau stain solution to verify the transfer was successful. Next, the membrane was washed in PBS-Tween 0.1% to remove the stain. Then the membrane was blocked with 5% skim milk solution for 1 hr and washed three times. After blocking, the membrane was incubated with shaking primary antibody anti-CYB5R3 (1:2000 diluted with PBST-BSA 5%) overnight at 40℃ and washed three times. Finally, the membrane was incubated with shaking for 1 hr with secondary antibody-HRP (1:5000 dilute with PBST-BSA 5%), washed three times, and stained with DAB substrate to visualize the results.

The reduction activity of purified diaphorase to metCygb

A total of 50 µg of Cygb was added to 1 µL of 1 mM K3Fe (CN)6 solution. The mixture was homogenized slowly and allowed to stand for 1 min to form a yellowish Cygb-Fe3+ solution. Then 50 µg of the diaphorase was added to the solution. The absorbance was read at a maximum wavelength of 426 nm for deoxyCygb and 416 nm for metCygb every 5 min for 2 hr (15). The reducing capacity of diaphorase against metCygb was determined by the following equation.

$$Reducing\ capacity\ (\mu g min^{-1}) = \frac{\left([metCygb]_i - \frac{A426_D}{A426_C}\right) - [metCygb_i]}{t}$$

$[metCygb]_i$ is the initial concentration of metCygb, $A426_D$ and $A426_c$ are absorbances at 426 nm of diaphorase and control when saturation occurs, and t is the time required for saturation to occur.

Results

Isolation and purification of NADH-diaphorase from bovine erythrocytes

The NADH-diaphorase purification process is shown in Table 2. While the specific activities and purification levels were similar in both peaks, we further purified peak B.

Table 2. Met-hemoglobin reductase activity during the purification process

Source	Volume (mL)	Protein concentration (mg/mL)	Activity (U/mL)	Specific Activity (U/mg)	Purification levels (Times)
Hemolysate	30	1480.31	4.526	0.003	-
Hemoglobin degraded, ammonium sulfate fractionated, and dialysis					
Dialysate	18	1.378	1.085	0.787	1
Gel filtration chromatography (n=3)					
Eluate A	4.5	0.07±0,01	0.31	4.292	5.45
Eluate B	6	0.24±0.01	1.209	5.034	6.4
Cation exchanger chromatography (n=2)					
Eluate B1	5.25±1.02	0.117±0.02	0.992	8.479	10.77
Eluate B2	4.5	0.05±0.1	0.124	2.48	3.15
Eluate B3	10.5±1.46	0.065±0.12	0.279	4.29	5,45

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Figure 1 shows two peaks. The fractions forming peak A contained 0.07±0.01mg/mL protein, whereas peak B (Soret) contained 0.24±0.01 µg/mL protein. Peak B had greater specific activity than peak A (Table 2), indicating it contains diaphorase. Peak B eluates were pooled and further purified by cation exchanger chromatography.

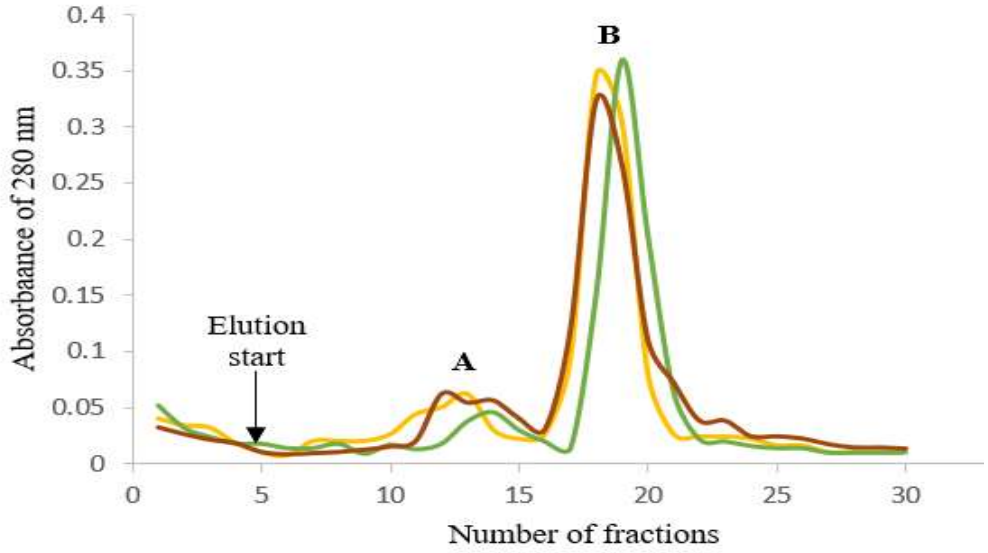

Fig. 1. Diaphorase purification by gel filtration chromatography (0,1 M PBS pH 6,8 was used as elution buffer).

Figure 2 showed three peaks. As shown in Table 2, peak B1 (Soret) had greater protein concentration than peak B2 or B3. Peak B1 peak had the greatest specific activity with 8.479 U/mg protein and was purified 10.77-fold greater than the dialysate as crude extract. The peak B1 fractions were pooled and stored for further analysis.

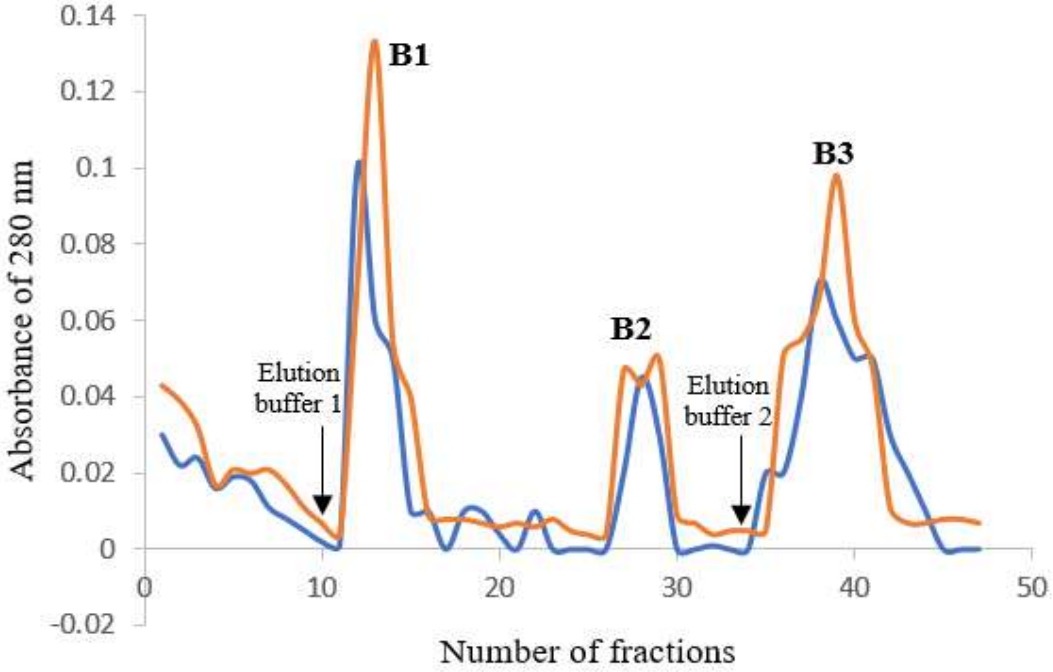

Fig. 2. Diaphorase purification by cation exchange chromatography (Elution buffer 1 was 0.05 M PBS pH 6.8, and elution buffer 2 was 0.05 M PBS pH 6.8 contain 0.25 M NaCl).

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SDS-PAGE and WB

Protein bands of 27 and 30, 60-75, and 100-150kDa were seen in the hemolysate. Bands of27-30 kDa and 60-75 kDa were seen in the dialysate. Bands of 30.09 kDa were seen in eluate B and eluate B1 (Fig. 3).

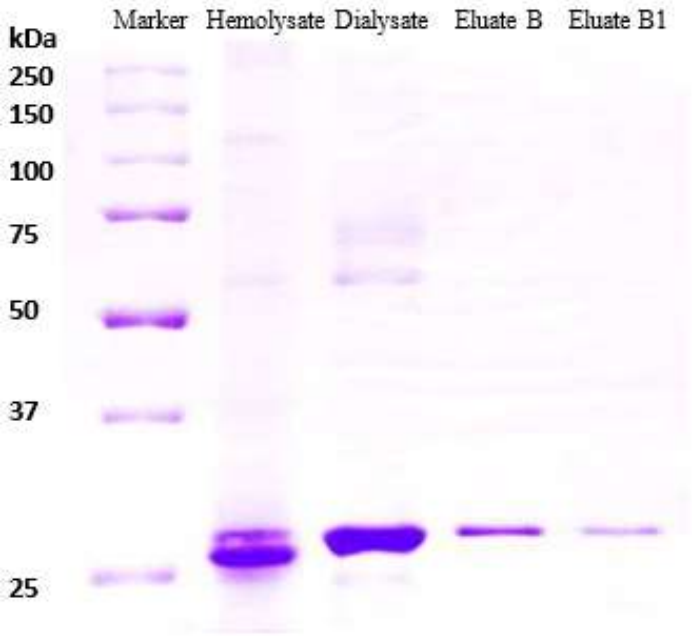

Fig. 3. SDS-PAGE of diaphorase (isolation-purification process) and cyb5r3 stained with CBB.

Proteins from the gel were transferred onto nitrocellulose and immunostained with anti-cyb5r3. The membrane was then incubated with an HRP secondary antibody and the band was visualized with diaminobenzidine (DAB). The anti-cyb5r3 reacted positively with eluate B1 containing diaphorase and cyb5r3 at 30.09 kDa (Fig. 4).

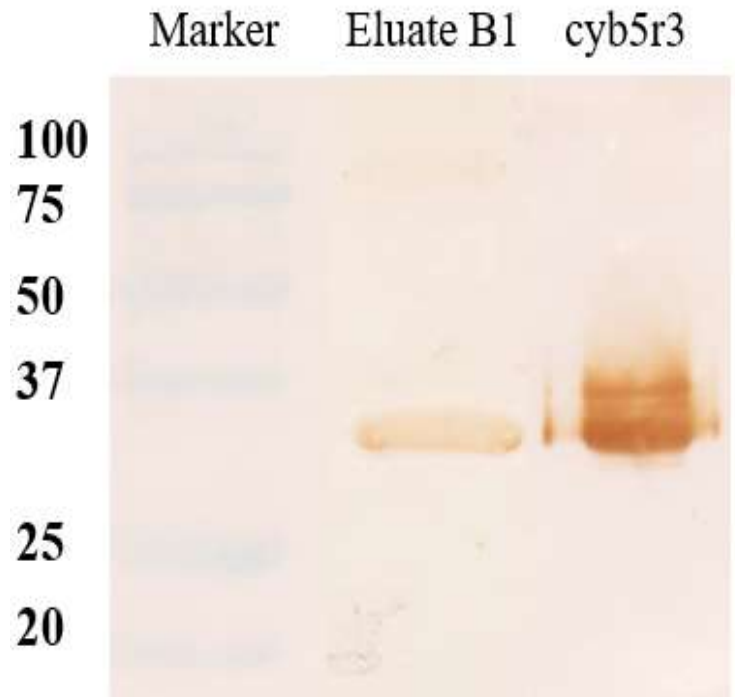

Fig. 4. Western blot of diaphorase (eluate B1) and cyb5r3 immunostained with anti-cyb5r3 and visualized with DAB staining.

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Kinetic parameters of purified diaphorase to metHb and metCygb

Figure 5A presents the reducing properties of purified diaphorase to reduce various concentration of metHb (0,125 – 2 g/dL) to deoxyHb. During the observation, we noticed increased specific activity with the increased metHb concentration. Km and Vmax values were obtained by inversion the Michaelis-Menten equation, and the inverted data were plotted in Fig. 5B.

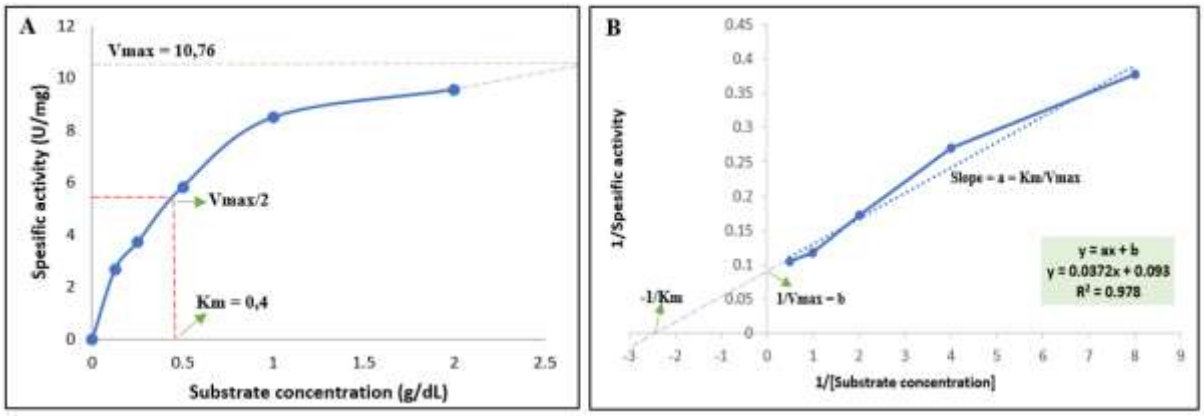

Fig. 5. A. Substrate concentration vs. specific activity to show the Vmax and Km values
Fig. B. Lineweaver-bulk plot (1/substrate concentration vs. 1/specific activity) to calculate Km and Vmax from the linearity equation.

To assess the ability of purified diaphorase to reduce metCygb (Cygb with Fe^{3+}), Cygb had to be readily oxidized. The optical density of deoxyCygb was observed for 120 min.

DeoxyCygb absorption increased for 40 minutes, and then decreased gradually over the following 80 min. After 140 min the absorbance was still greater than the initial absorbance (0.426 vs. 0.28 absorbance unit) (Fig. 6)

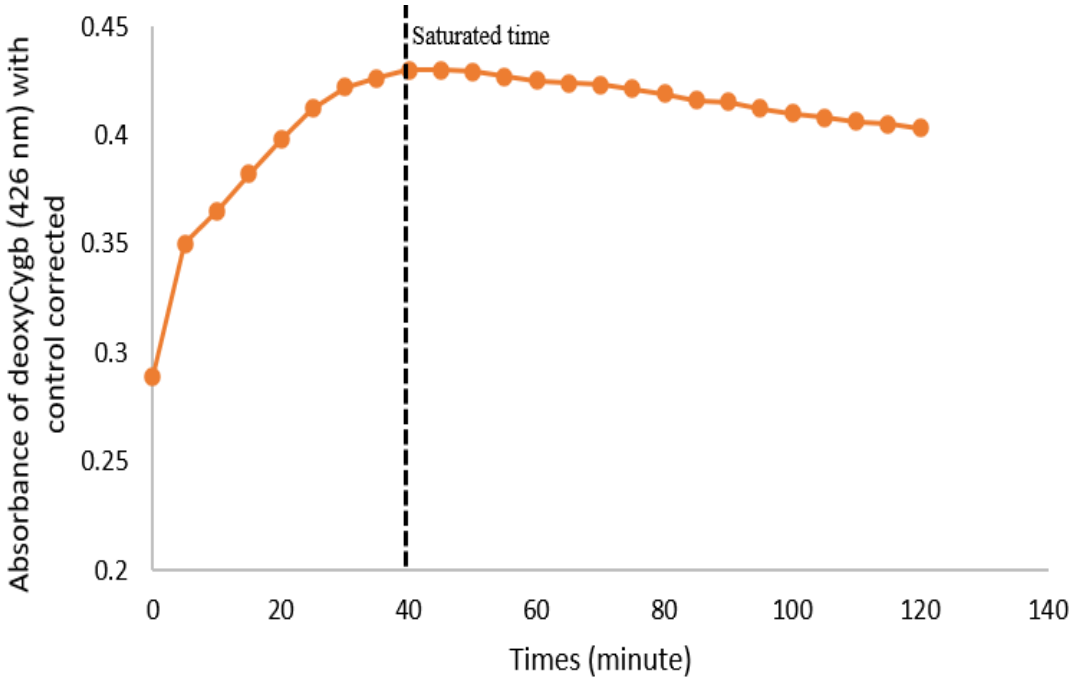

Fig. 6. DeoxyCygb absorbance over time when reduced by purified diaphorase.

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Discussion

Diaphorase was first discovered by Adler et al. around 1940. They identified two types of diaphorase from heart muscle and determined it could catalyze hydrogen transport from dihydrocodehydrogenase I to some acceptors, excluding oxygen (8). Some research has isolated a diaphorase from human erythrocytes and it is believed to reduce metHb. Huenneken et al. reported that the diaphorase could reduce oxidized triphosphopyridine nucleotide with oxygen, metHb, or cytochrome c as a terminal electron acceptor (9). Scott et al. concluded that diaphorase activity is decreased in methemoglobinemia patients (10). Hultquist et al. confirmed that diaphorase was a metHb reductase, and they said that cytochrome b5 was an effective electron acceptor (6). In the 1970s, diaphorase was established as cytochrome b5 reductase. Hagler et al. were discovered that cytochrome b5 reductase from bovine heart could reduce metMb (12).

Redox reactions take place simultaneously in the body, especially in the mitochondria, which play a major role in the process of oxidative phosphorylation, one of process that contributes much of ROS products. This simultaneous redox process indicates oxidative environment in our body that possible to oxidize intra and extracellular proteins, especially redox sensitive proteins such as globin proteins containing the transition metal Fe (2,3). Around 1.5-3% oxyHb or oxyMb oxidized naturally due to the structural rearrangement (6). Besides naturally oxidation, oxizided Cygb is also obtained due to its function as like as NO dioxygenases. Oxyheme of Cygb, $Fe^{3+}(O_2^-)$ have greater affinity against nitric oxide free radical (NO•) than deoxyheme of Cygb. It makes heme losses the oxygen and converts NO• into stable nitrate (NO_3^-) that leaving heme in ferric (Fe^{3+}) form (5,13). Formation of metglobin indicated by color changing (red to brown) or absorption wavelengths shifting (Table 3).

Table 3. Wavelength shifting on oxy, deoxy, and met form of globin protein

Protein	Form	Maximum wavelength (nm)
Hemoglobin (5)	Oxy	542.2 ± 0.19 (~540) 577.0 ± 0.06
	Deoxy	553 ± 0.21
	Met	631.8 ± 0.21 (~630)
Myoglobin (21,22)	Oxy and deoxy	405 (soret), 500, 575, 630, 739
	Met	Loses peak at 575
Cytoglobin (15)	Oxy	420 (soret)
	Deoxy	426 (soret)
	Met	416 (soret)

Unlike Hb or Mb, Cygb has a heme group with hexacoordinate (hx) bond. Fe ion in hem group has six sites that ligands can occupy. The four Fe sites have attached to the N atom of pyrrole groups. The fifth site is occupied by proximal histidine (HisF8). In Hb and Mb, the sixth site is a free and can bind exogenous ligands easily. But Cygb's sixth site is bound to distal histidine (HisE7) in both the Fe^{2+} and Fe^{3+} states (14, 23). The heme conformation differences might be leads to the lower diaphorase's reducing activity on Cygb, compared to the reducing activities against metHb, its natural substrate. Some literature investigators said that several hxglobin have a constant affinity to oxygen transport, but the dissociation rates are too slow because of much faster autooxidation rates than

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pentacoordinate globin. The low diaphorase's reducing activity profile on metCygb might be due to autooxidation and ligand dissociation rates. But the reasons for that phenomenon are not well understood (23–31).

In conclusion, the diaphorase (30.09 kDa) was successfully purified from bovine erythrocyte based on reduction kinetic of metHb, SDS-PAGE, and WB results. The diaphorase shows the specific activity to reduce bovine metHb of 8.479 U/mg with Km value 0.4 $\mu gmin^{-1}$ and Vmax was 10.76 $\mu gmin^{-1}$. However, it can only reduce 0.785 $\mu gmin^{-1}$ of metCygb (13.7 times lower than metHb). Furthermore, the reduction profile of metCygb was attained after 40 minutes of reaction. Therefore, we conclude that diaphorase did not significantly reduce metCygb rather than metHb, a natural substrate in cells. It suggested that metCygb reductase would not be identical with diaphorase.

Acknowledgements

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