The Enzyme that Reduces Oxidized Cytoglobin in Bovine Liver: An Exploration

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Abstract*—Oxidized cytoglobin (Cygb) can be reduced by supernatant of bovine liver cell homogenate as demonstrated by increased production of Cygb-Fe^{2+} from Cygb-Fe^{3+}. We hypothesized that, in bovine liver tissue, there is a protein that acts as reductase, which is analogous to diaphorase, acting on methemoglobin (metHb), and analogous to cytochrome b5 reductase 3 (CYB5R3), acting on metmyoglobin (metMb). The aim of this study is to explore the enzyme that can reduce oxidized Cygb to reduced Cygb. The putative enzyme in bovine liver homogenate was isolated using RIPA lysis buffer, purified by Cibacron blue chromatography, and confirmed by SDS-PAGE and western blot. The activity of the suspect reductase enzyme was determined by the ratio of maximum absorbance between Cygb-Fe^{3+} (metCygb) and Cygb-Fe^{2+} (deoxyCygb). We discovered that the reducing capacity of fraction, purified by Cibacron blue chromatography, was weaker than the cell extracted from bovine liver. The gel electrophoresis analysis indicated that the Cibacron blue fraction molecular weight was ~50 and ~60 kDa, whereas CYB5R3 was 34 kDa. Moreover, metcytoglobin could not be reduced by diaphorase. We concluded, there is a reductase enzyme in the cell extract that can reduce Cygb-Fe^{3+} to Cygb-Fe^{2+}; however, it is neither diaphorase nor CYB5R3.

***Index Terms*—chromatography, cytoglobin, purification, enzyme, reductase**

I. Introduction

Globins are heme-containing proteins and ubiquitously expressed in vertebrates, where they serve a broad range of functions, mainly in reversible oxygen (O_2) binding. Thus, they are important in the respiratory system of living species [1]. Hemoglobin (Hb) and myoglobin (Mb) can reversibly bind O_2. Mb is found in muscle cells and can store, transport, and deliver O_2 to mitochondria [2], [3]. Overall, the coordination of Hb and Mb results in the delivery of O_2 from the respired air to intramuscular mitochondria [2].

The iron in Hb and Mb can undergo oxidation from Fe^{2+} to Fe^{3+}, in which neither state (metHb and metMb forms) nor globin can bind O_2 [4]-[7]. To restore the O_2 binding function, Fe^{3+} must be reduced to Fe^{2+}. Erythrocytes have two enzymes: (a) NADH methemoglobin reductase (diaphorase) and (b) methemoglobin NADPH-flavin dependent reductase, which can reduce Fe^{3+} in metHb to Fe^{2+} [8]. More recently, the analogous enzyme was identified for reducing metMb (Fe^{3+}) in bovine heart muscle. The enzyme, known as metMb reductase, apparently is the same as cytochrome b5 reductase [9]. Later, it was also discovered that diaphorase and cytochrome b5 reductase are the same molecule [10].

Cytoglobin (Cygb), another type of extra-erythrocyte globin, is ubiquitously found in non-muscular and neuronal tissues. The function of Cygb is still unclear; several properties of Cygb have been proposed, namely intracellular O_2 storage, NO scavenger, peroxidase, and tumor suppressor [11]. However, as the molecule has a globin structure, namely a heme ring, sandwiched by peptide folds of a globin, it can be expected that one of its main functions is to reversibly bind O_2 to allow its iron to undergo oxidation, which will hamper the O_2-binding function. It has been demonstrated that iron can be oxidized to form metCygb, which can be reduced to Cygb by vitamin C [12]. We hypothesized that reduced enzyme exist in the cell extract. The objective of this study was to investigate the presence of a putative enzyme in the same tissue extract and to compare its properties with those of metHb reductase or diaphorase.

II. Material and Methods

A fresh bovine liver was obtained from a slaughterhouse in Jakarta as research material. The extraction stages were performed using RIPA lysis buffer. Cibacron blue affinity chromatography was used to purify

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the suspect enzyme, confirmed by SDS-PAGE and western blot (WB).

A. Extraction

The fresh bovine liver tissue was chopped and then homogenized by a homogenizer. The RIPA lysis buffer solution (ChemCruz® sc-24948) was added and mixed by vortex; the mixture was then incubated in ice for 30 minutes. At the end of the incubation period, the mixture was centrifuged at 10,000x*g* for 10 minutes at 4°C. The supernatant obtained was total cell lysate, which was stored at -80°C.

B. Affinity Chromatography

Cibacron blue® (Bio-Rad® Cat. No. 153-7302) was equilibrated with 0.03 M PBS pH 6.0, containing 0.001 M EDTA. One hundred µL of lysate was applied to the column and washed with the same buffer until the A280 nm was less than 0.002. Then the buffer was replaced with 0.03 M PBS pH 6.0, containing 0.001 M EDTA and 1 M NaCl until the A280 nm was less than 0.002. The fractions formed peaks, which were collected for further analysis.

C. SDS-Polyarylamide Gel Electrophoresis (PAGE) and WB

SDS-PAGE was performed under two conditions. For the first condition, the sample was denatured in a sample buffer, containing β-mercaptoethanol for denaturating all the proteins. In another analysis, we carried out the natural SDS-PAGE, using the same sample buffer but without β-mercaptoethanol. The electrophoresis was carried out for 60 minutes in 150 V; the gel was then stained by silver stain. The WB analysis was performed by electro-transferring the proteins in the gel electrophoresis in the nitrocellulose membrane. The electro-transfer was performed for 30 minutes in 25 V. After the transfer, the nitrocellulose membrane was soaked in Phosphate Buffer Saline (PBS)-Tween, containing 5% skim milk, for blocking all the free site in the membrane. The process was allowed to stay overnight at 4°C. After the blocking, the membrane was shaken and soaked in PBS-Tween, containing anti-CYB5R3 antibody (SantaCruz® sc398043) for an hour, followed by the second antibody, goat anti-mouse IgG2a conjugated HRP (Abcam® ab97245), for an hour; this was immediately followed by the addition of the substrate. The images were recorded by Biomolecular imager.

D. MetCygb Reducing Capacity Test

To 50 µL recombinant Cygb (Abcam® ab97347) solution in a micro cuvette, 1 µL of 1 mM $K_3Fe(CN)_6$ was added. The optical density of the mixture was measured after one minute. After one minute, optical density was scanned from 400-600 nm. The maximum optical density was noted as the optical density of metCygb. The experiment was performed to elucidate the reducing capacity of lysate, Peak A, Peak B and diaphorase form *Clostridium kluyveri* (Sigma® D5540-100UN) for reducing oxidized Cygb. Fifty microliters of each sample were added to the metCygb solution. Optical density was measured at 416 nm (Cygb-Fe^{3+}) and 426 nm (Cygb-Fe^{2+}). The observations continued and optical density was measured every five minutes for two hours.

III. Results

A. Affinity Chromatography

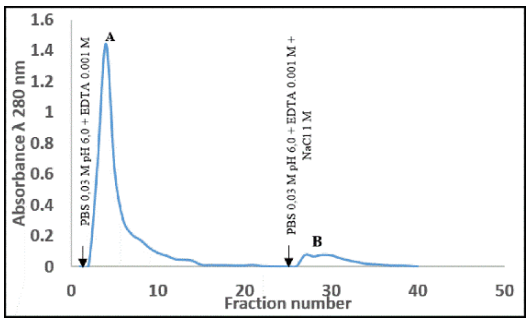


Figure 1. The result of reductase purification by Cibacron blue affinity chromatography.

Fig. 1 showed two peaks. Peak A has the highest absorption in fraction number 4, with a protein concentration of 2311.79 µg/mL, and Peak B has the highest absorption in fraction number 27 with a protein concentration of 313.5 µg/mL.

TABLE I. Total Protein in Purification of Reductase Enzyme from Bovine Liver

Sample	Protein Concentration
Lysate of bovine liver	5790 µg/mL
Peak A	2311.79 µg/mL
Peak B	313.5 µg/mL

Table I shows the protein content of the entire isolation process and the purification of enzyme reductase from bovine liver.

B. SDS-PAGE

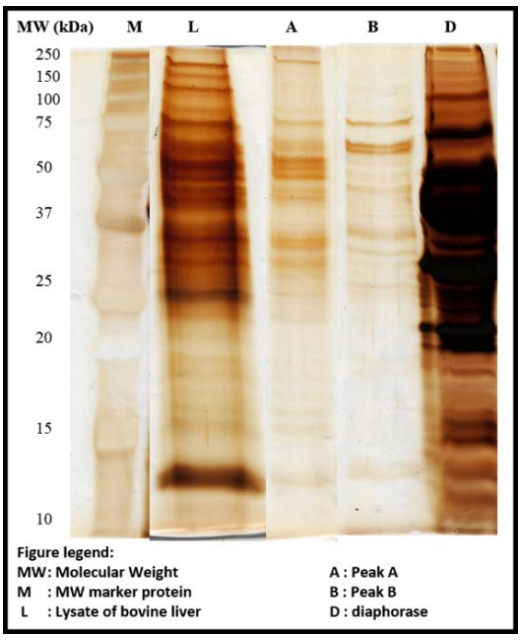


Figure 2. SDS-PAGE of lysate of bovine liver (L); Cibacron blue affinity chromatogprahy fraction (peak A, B); and diaphorase stained with silver.

Fig. 2 shows SDS-PAGE of the total cellular protein extracted using RIPA lysis buffer, before and after affinity chromatography molecular weight marker proteins. Lane L represents the total lysate protein, Lane A shows the not-retained proteins, and Lane B represents the retained proteins. Lane A contains several proteins (20 proteins) after spectral analysis, and Lane B, as expected, contains fewer proteins than Lane A. However, to our surprise, we found that more than one protein was bound by Cibaron blue Affi gel. The range of MW in Lane A is from ~12–240 kDa. The main protein has a molecular weight of ~50 kDa. In Lane B, the MW range is from ~12–75 kDa and the main protein is about ~60 kDa.

We also did SDS-PAGE in the semi-denatured condition, namely a condition in which the protein samples were mixed with SDS but without mercapto-ethanol and heating. The results are presented in Fig. 3. The analysis was conducted because, in SDS-PAGE, the denatured condition in which mercapto-ethanol is added, any oligomeric protein will split into their subunits. Consequently, it would be difficult to identify which protein has specific functions. Lanes A and B in Fig. 3 contain fewer proteins than in denaturating condition.

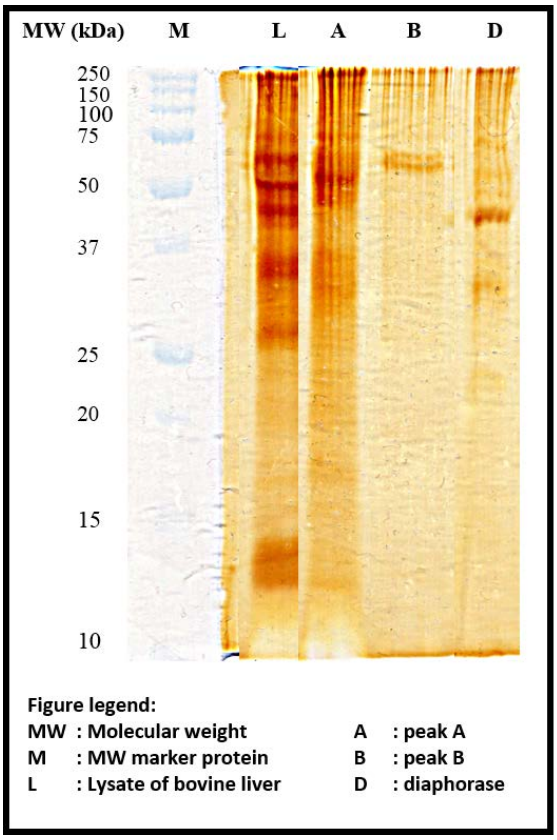


Figure 3. SDS-PAGE analysis in semi-denatured condition.

The MW of common protein in Lanes L and A is about ~50 kDa. Other than that, we found the same band protein in Lanes L and B with MW being ~60 kDa, whereas the band protein in Lanes L and D indicates an MW of ~40 kDa. The band protein in Lanes A, B and D are not the same, as seen in Fig. 3.

C. WB

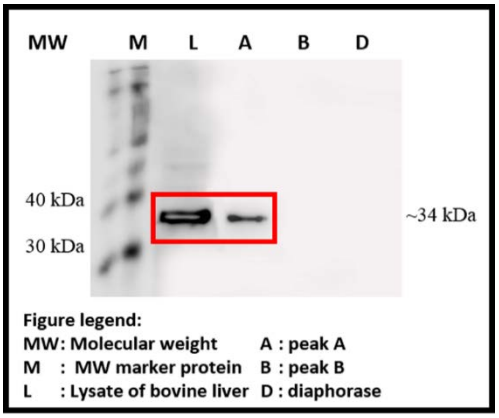
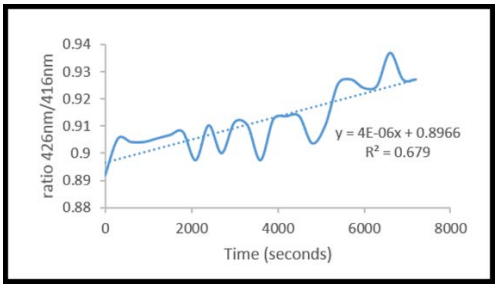
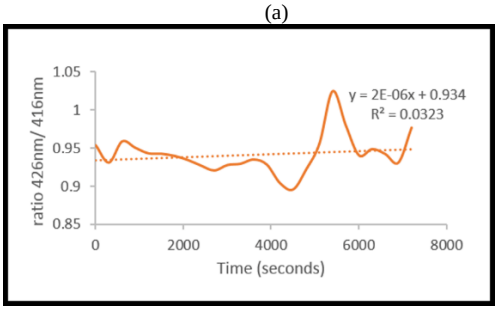


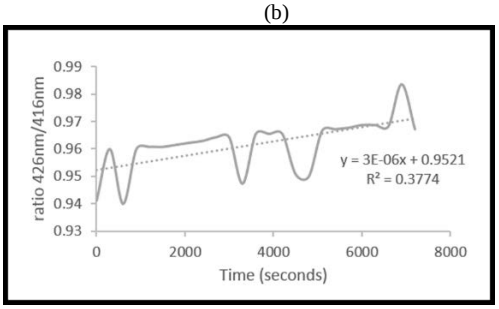
Figure 4. WB analysis of protein in purification of reductase enzyme from bovine liver.



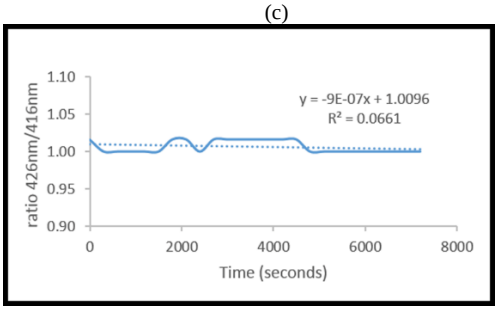
(a)



(b)



(c)



(d)

Figure 5. MetCygb reducing capacity assay of (a) lysate of bovine liver; (b) Peak A; (c) Peak B; and (d) diaphorase.

The WB analysis was conducted using anti-CYB5R3 antibody as probe (see Fig. 4). It was found that the WB is positive in lysate (Lane L) and slightly in Peak A (Lane A). The position of the band is about 30-40 kDa. Lane B, containing retained Cibacron blue gel affinity, and Lane D, containing diaphorase, do not show a positive WB reaction.

D. MetCygb (Cygb-Fe^{3+}) *Reducing Capacity Assay*

All the experiments performed in micro cuvette were placed in spectrophotometer. To demonstrate the reducing capacity of each peak, Cygb had to be oxidized. The optical density ratio between Cygb-Fe^{2+} and Cygb-Fe^{3+} was calculated, and the results can be seen in Fig. 5.

Fig. 5a presents the reducing properties of bovine liver lysate to reduce metCygbFe^{3+} to CygbFe^{2+}. During the observation, we noticed an increase in CygbFe^{2+} optical density (R^2 = 0.679). The reducing properties of Peak A can be seen in Fig. 5b. Despite the increase in the optical density of CygbFe^{2+}, the R value is very low (R^2 = 0.0323). Fig. 5c shows the reducing capacity of Peak B. In this part, we noticed that the reducing capacity was much higher than Peak A and nearly half the lysate reducing capacity. In Fig. 5d, the diaphorase shows no reducing capacity at all.

IV. Discussion

Cytoglobin was first discovered by Kawada in 2001, isolated from liver stellate cell and named as STAP (stellate cells activated associated protein) [13]. The identity of STAP as a member of hemoglobin family was made by Burmester et al. in 2002 who called the newly found globin protein "Cygb" [11]. Until now, despite ubiquity, the definitive function of this widely distributed hemoglobin is still unclear. Although the specific function of Cygb cannot yet be assured, most of the proposed properties are related to O_2 binding [14], [15]. To realize this function, as indicated by hemoglobin and myoglobin, the heme iron must be maintained in the reducing state (i.e., in Fe^{2+} form) [6], [7]. Once Fe^{2+} is oxidized to Fe^{3+} form, the Hb cannot bind O_2 anymore. In erythrocyte Hb and in myoglobin, this form (known as metHb and metMb) are reduced back to Fe^{2+} form. These reactions need specific enzymes, methemoglobin reductase and metmyoglobin reductase [9], [10]. Several spectral studies demonstrated that Cygb can be oxidized to metCygb and can be reduced by vitamin C [16], [17].

We treated the bovine liver homogenate with RIPA lysis buffer. We used the lysis buffer in order to obtain the maximum total intracellular protein. The lysate was applied directly to the Cibacron blue affinity chromatography and we found two peaks. The Cibacron blue gel binds any protein or enzyme with a compound containing at least two cyclic moiety as co-factor, mainly protein or enzyme using purine nucleotide (e.g., NAD and NADP). There are a number of enzymes, especially the hydrogenases, which use NAD or NADP as co-enzyme. It can be understood that the eluate of post-Cibacron blue purification (Peak B) contains several bands of protein.

The reducing capacity of lysate, Peak A, Peak B and diaphorase, in the oxidized Cygb was studied by spectral analysis. The reducing capacity can be seen in lysate (Lane L) and relatively clearly in Peak B (Lane B). Peak A, containing materials not retained by affinity column, showed a very weak reducing capacity. On the other hand, diaphorase actually enhanced the Cygb-Fe^{2+} product. From this point of view, it can be suggested that Peak B may contain an enzyme that can reduce metCygb to Cygb with an MW of ~60 kDa.

The identity of protein was tested with anti-CYB5R3 antibody using WB. It was found that the antibody bound firmly and met a single in Lysate (Lane L) and Peak A (Lane A) with an MW of ~34 kDa. The anti-CYB5R3 antibody cannot bind any protein in Peak B (Lane B) and diaphorase (Lane D). All of the results suggest that, in the liver cells, there should be an enzyme that can reduce the oxidized Cygb. Apparently, the enzyme needs nicotinamide nucleotide as co-enzyme. Our finding of the hypothetical enzyme, known as metCygb reductase, is different from metHb reductase in erythrocyte and metMb reductase from bovine heart muscle. The MW of diaphorase from *Clostridium kluyveri* is about ~24 kDa [18]. It is much lower than our enzyme with an MW of ~60 kDa and a metMb reductase of ~34 kDa. The immunochemical test also shows very clear differences. The anti-CYB5R3 antibody bound a protein in lysate and Peak A but not in Peak B. We suggest that metCygb reductase is not identic to metHb reductase; it is very different from metMb reductase, which identified with CYB5R3.

V. Conclussion

In summary, the liver cells may contain an enzyme that can reduce oxidized Cygb with no immunochemical properties of CYB5R3 as metMb reductase. On the other hand, diaphorase cannot reduce metCygb and it cannot be bound by anti-CYB5R3 antibody either. We propose performing the purification of the enzyme through other approaches, such as ligand affinity chromatography (i.e., NAD) in combination with anion exchange chromatography.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Nabilla Sonia Sahara conducted the research; Mohamad Sadikin and Sri Widia A Jusman analysed the data; Nabilla Sonia Sahara, Mohamad Sadikin and Sri Widia A Jusman wrote the final paper.

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