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Original Article

Isolation and Characterization of Neuroglobin and The Reducing Enzyme Metneuroglobin (Neuroglobin Fe3+) From Bovine Brain Tissue

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ABSTRACT

Background/Aim: The brain uses 20% of the O2 consumed by the body for energy metabolism. In 2000, found a protein that is thought to be a binding O2 in the brain, namely neuroglobin (Ngb). Ngb is a member of the hemoprotein which has a heme group. The iron ion in the haem group can be oxidized, so a reducing enzyme is needed. In this study, the isolation, purification, and characterization of Ngb protein and the reducing enzyme from oxidized neuroglobin (neuroglobin Fe3+) were carried out. **Materials and methods:** Ngb protein was isolated by fractionation technique using ammonium sulfate 90% saturation, purified by anion exchange chromatography (DEAE Cellulose) and immunoaffinity chromatography, confirmed by SDS-PAGE and Western blot. The metneuroglobin-reducing enzyme was isolated by RIPA lysis buffer, purified by Affi gel blue chromatography, and confirmed by SDS-PAGE. **Results:** The isolated Ngb obtained has a molecular weight of 17.26 kDa. Spectrum analysis in the wavelength range of 350-500nm, showed the afternoon peaks of deoxyNgb, oxyNgb, carboxyNgb and metNgb were 415 nm, 405 nm, 405 nm, and 420 nm, respectively. The results of the isolation of the reducing enzymes obtained consisted of 2 parts, namely the matrix-bound eluate (eluate-1) and matrix-bound eluate (eluate-2). SDS-PAGE results of eluate-1, eluate-2 and Ngb-free fraction (byproduct of Ngb purification) showed the same 3 bands at a molecular weight of 72.45; 26.84 and 16.33 kDa were suspected as reducing enzymes. **Conclusion:** The reduction kinetics was tested by reacting the fraction and metNgb and measuring the deoxyNgb uptake formed per unit time. The results of the measurement of the ratio of NgbFe3+ to NgbFe2+ from the free fractions Ngb, eluate-1 and eluate-2, which has the best reducing activity is eluate-1 because it has the best regression value of 0.8769.

Key words: Neuroglobin, Reductase enzyme, Neuroglobin absorption spectrum, Bovine brain tissue.

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INTRODUCTION

One organ with a high rate of ATP demand is the brain. Unlike most tissues, which can rely on anaerobic metabolism to produce ATP in the absence of oxygen (O2), the brain cannot do so. At rest, the brain consumes 20% of the oxygen and 50% of the glucose consumed by the body. If the brain does not receive O2 for more than 5 minutes or its glucose supply is interrupted for more than 15 minutes, it is damaged. As a result, the brain is completely reliant on a constant supply of O2 and glucose.

Neuroglobin, an oxygen-binding protein discovered in the brain in the early 2000s (Ngb). Ngb is found in the central nervous system, peripheral nervous system, endocrine system, and retina. 1 Ngb is made up of 151 amino acids and has a molecular weight of about 17kD. The polypeptide chain folds into eight distinct -helical segments (A-H segments) (3-over-3 helical sandwich structure). Ngb is thought to function as an O2 transport protein, facilitating O2 diffusion into mitochondria in brain cells for aerobic respiration, as well as a NO scavenger.

In contrast to hemoglobin (Hb) and myoglobin (Mb), Ngb has a heme structure similar to cytoglobin (Cygb) i.e. hexacoordination in which the sixth coordination site is occupied by a HisE7 residue distal to the globin chain. Therefore, in order to bind to an external ligand there must be a cleavage of the bond with the distal HisE7. The breaking of the bond could be due to the greater affinity of the ligand to the distal HisE7 residue and the conformational change in the heme group.[3,4] The conformational change in the heme group can affect the absorption spectrum. Dewilde (2001) stated that there are differences in the absorption spectrum when Ngb is not bound to oxygen (deoxyNgb), when it is bound to oxygen (oxyNgb) and when it is bound to carbon monoxide (carboxyNgb) did by Syailendra.[5]

The iron ion (Fe2+) in the globin protein's heme group can be oxidised to Fe3+, which is denoted by the prefix meth. The globin protein cannot bind O2 in this state, which is extremely dangerous for cell survival. MetHb reductase or diaphorase is found in erythrocyte cells to keep Fe in a Fe2+ state. Similarly, enzymes in liver tissue can reduce Fe3+ from oxidised Cygb. According to Sahara (2020), oxidised Cygb could be reduced by the homogenate supernatant of beef liver cells, as evidenced by increased production of Cygb-Fe2+ from Cygb-Fe3 Given that Ngb is a group of globin proteins that have the same heme structure, namely hexacoordination, there is a possibility that Ngb also has a reducing enzyme capable of reducing oxidised Ngb. As a result, this study was carried out to see if the Ngb-free brain tissue extract isolated from bovine brain tissue could also reduce Fe3+ from metNgb to Fe2+ again. 6,7

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MATERIALS AND METHODS

This research is an experimental study using fresh cow brain tissue as the research sample. The research includes the isolation of Ngb protein and the isolation of the reductase enzyme that may be able to reduce Ngb from cow brain tissue. The isolation process goes through the stages of separation, purification, and characterization.

At the Ngb protein separation stage, homogenization, salting-out, and dialysis processes were carried out. In the purification stage, diethylaminoethyl cellulose (DEAE) column chromatography and immunoaffinity column chromatography using anti-Ngb antibodies were performed. The purification results were confirmed using the western blot method. Furthermore, in the characterization stage, an absorption spectrum analysis of Ngb was carried out under several conditions, namely bound to O_2 (oxyNgb); not bound to O_2 (deoxyNgb); bound to CO (NgbCO); oxidized (metNgb); and under reduced conditions by addition of ascorbic acid and neuroglobin-free eluate.

The isolation stage of the reductase enzyme against neuroglobin from fresh beef brain tissue, went through the stages of separation, purification, confirmation, and characterization. The separation step was carried out using a buffered Radioimmunoprecipitation assay (RIPA) lysis solution. Furthermore, the purification stage was carried out by Affi-gel® blue affinity column chromatography. In the characterization stage, the oxidized Ngb was reduced by purified reductase enzyme and assessed by measuring its absorbance using a spectrophotometer.

Measurement of total protein content of beef liver dialysate using the Christian-Warburg method using a standard curve of Bovine Serum Albumin (BSA) solution made with a concentration range of 25, 50, 100, 200, 300, 400, 500 and 1000µg/mL. The BSA solution and sample were measured for absorbance at λ 280nm in duplicate for each test solution. Measurement of protein content in beef liver dialysate was obtained from the results of calculations based on the regression equation from the standard curve of BSA solution.

Neuroglobin spectrum analysis (DeoxyNgb Absorption, OxyNgb Absorption, CarboxyNgb Absorption, MetNgb Absorption)

Spectrum analysis of deoxyNgb was carried out with 100 L Stokes reagent solution added a few drops of concentrated NH4OH solution until the precipitate formed dissolved. Three drops of the solution was added to a test tube containing 50 L Ngb. Next, the OxiNgb spectrum was formed to form deoxyNgb. Furthermore, into the test tube carbon monoxide gas flowed for approximately one minute. Spectrum analysis of CarboxyNgb by means of 50µL Ngb was put into a test tube and added 1µL solution of $K_3Fe(CN)_6$ 1mM. Slowly the mixture was homogenized and allowed to stand for 1 minute. Then perform an absorption scan on a spectrophotometer with a wavelength range of 400-600nm.

Determination kinetic reduction of Ngb-Fe^{3+} to Ngb-Fe^{2+}

A total of 50µL of recombinant Ngb was added to 1µL of 1mM $K_3Fe(CN)_6$ solution in the cuvette. Slowly the mixture was homogenized and allowed to stand for 1 minute to form a metNgb solution. Then 50 L of ascorbic acid was added to the metNgb solution. Furthermore, readings were taken at the maximum wavelength of metNgb and deoxyNgb every 5 minutes for 2 hours.

RESULTS

Isolation and purification of neuroglobin protein

From 100 grams of homogenized fresh beef brain, 160 mL of homogenate was obtained. The homogenate was then centrifuged and 55 mL supernatant was obtained. The supernatant was fractionated by adding ammonium sulfate with a saturation concentration of 90%. The result of fractionation as much as 19.411 grams was dialyzed using a cellophane membrane to separate ammonium sulfate from protein. The dialysis process lasted for 2 days, by changing the Tris-HCl 5 mM pH 8.5 buffer every day. The dialysis process was monitored by the sulfate ion test which was indicated by the absence of a white precipitate of barium sulfate ($BaSO_4$). The dialysis results obtained were 50 mL.

Bovine brain dialysate was purified by DEAE Cellulose anion exchange chromatography and immunoaffinity. The levels of Ngb protein from cow brain tissue in the separation process are shown in table 1.

The results of the Ngb protein molecular weight analysis using the SDS-PAGE electrophoresis technique can be seen in Figure 1.

From the SDS-PAGE electrophoresis image, it can be seen that the dialysate and D2 eluate of DEAE Cellulose anion exchange column chromatography produced many bands, while the I2 eluate of immunoaffinity column chromatography and recombinant Ngb produced one band with a molecular weight of 17.26 kDa. To ensure the presence of Ngb, a WB test was carried out as shown in Figure 2.

Isolation and purification of reductase enzymes

The results of protein isolation from 5 grams of bovine brain tissue using the RIPA lysis buffer kit lysis solution obtained as much as 500 µL of bovine brain cell extract. Based on the standard curve in Figure 2. The protein content of bovine brain cell extract was 29.53 mg/mL. Bovine brain cell extract was purified by Affi-gel blue affinity column chromatography. The protein levels of the Ngb reductase enzyme from cow brain tissue in the separation process are as follows in Table 2.

The results of the analysis of the molecular weight of the reductase enzyme using the SDS-PAGE electrophoresis technique can be seen in figure 3.

SDS-PAGE electrophoresis visualization showed that there were the same bands for Ngb-free eluate, eluate I, and eluate II at 72.45 kDa.

Table 1: The levels of Ngb protein from cow brain tissue.

Sample	Protein Concentration mg/mL
Cow brain homogenate	42,53
Dialysate F90% saturated	24,31
Results of D2 peak anion exchange chromatography (purification 1)	1929,78
Results of D2 peak anion exchange chromatography (purification 2)	1695,33
I2 peak immunoaffinity chromatography results (purification 1)	190,89
I2 peak immunoaffinity chromatography results (purification 2)	133,11

Table 2: Isolation and purification of reductase enzymes.

Sample	Protein Concentration mg/mL
Cow brain lysate	29,53
Peak affinity chromatography results A1 (purification 1)	666,44
Peak affinity chromatography results A1 (purification 2)	839,78
A2 peak affinity chromatography results (purification 1)	212
Peak affinity chromatography results A2 (purification 2)	167, 56
A1 peak-forming fraction pool	627,55
A2 peak-forming fraction pool	190,89

In Ngb-free eluate and II eluate, it can be seen that there are other components, while in eluate I there is only one single band. The band is thought to be a reducing enzyme, each of which will be tested for its ability to reduce Ngb.

Neuroglobin spectrum analysis

A total of 50 L Ngb was reduced to deoxyNgb with the addition of Stokes reagent. The scan results of the deoxyNgb absorption spectrum (orange color) in the wavelength range of 370-470 nm can be seen in Figure 4.A. and Scan results show that the maximum absorption of purified and recombinant deoxyNgb (blue color) is at a wavelength of 415 nm.

Ngb was reduced to deoxyNgb by adding Stokes reagent, which was aerated with O¬2 gas for 1 minute after measuring the absorption spectrum of oxyNgb in the wavelength range of 350-500 nm. The scan results show that the maximum absorption of purified oxyNgb is at a wavelength of 405 nm as shown in Figure 4.B. CarboxyNgb analysis was carried out by reducing Ngb to deoxyNgb by adding Stokes reagent, then aerating with CO¬2 gas for 1 minute. The results of scanning the absorption spectrum of carboxyNgb in the wavelength range of 350-500 nm. The scan results show that the maximum absorption of purified carboxyNgb is at a wavelength of 405 nm as shown in Figure 4.C. metNgb analysis was carried out with 50 µL Ngb oxidized to metNgb with the addition of 33% $K3F(CN)_6$ solution. The results of the scan of the metNgb absorption spectrum in the wavelength range of 350-500 nm can be seen in Figure 4.D. The scan results showed that the maximum absorption of purified and recombinant metNgb was at a wavelength of 420nm.

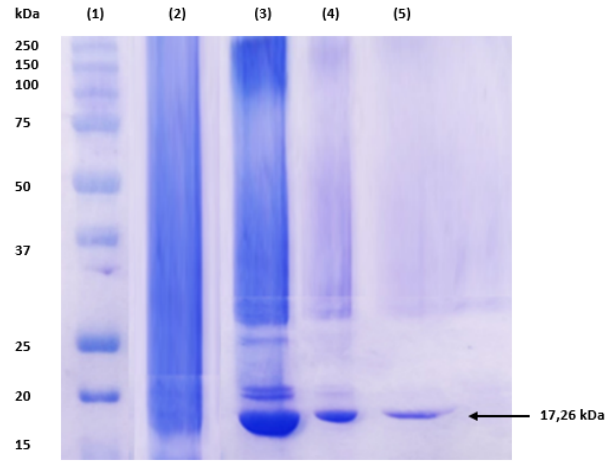

Figure 1: The results of the SDS-PAGE electrophoresis of Ngb protein.

Lane description (1) Standard molecular weight protein (2) Dialysate precipitated Ammonium sulfate fraction 90% (3) Eluate D2 anion exchange column chromatography DEAE (4) Eluate I2 immunoaffinity column chromatography (5) Recombinant Ngb.

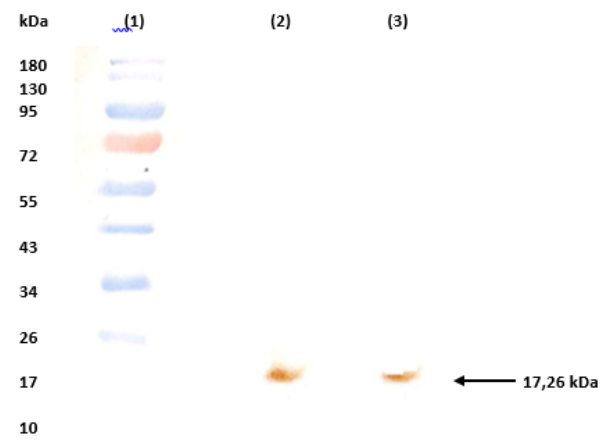

Figure 2: Western blot test results.

Row descriptions: (1) Protein molecular weight standard (2) Immunoaffinity column chromatography eluate (3) Recombinant Ngb.

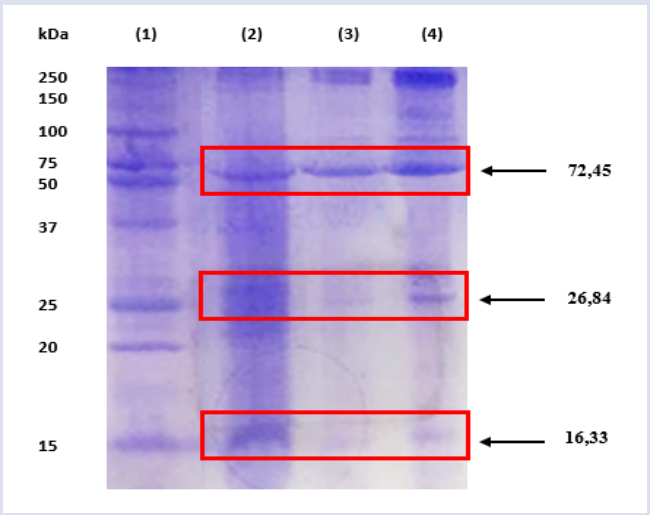

Figure 3: Results of SDS-PAGE electrophoresis of the enzyme reductase.

Row descriptions: (1) Protein molecular weight standard (2) Ngb-free fraction (3) Eluate A1 affinity column chromatography (4) Eluate A2 affinity column chromatography.

Kinetic reduction of NgbFe3+ to NgbFe2+

Reduction kinetics of NgbFe3+ which was reduced back to NgbFe2+ using isolates from cow brain tissue, metNgb reduction test was performed with Ngb-free fraction, the eluate was not bound to the affinity column matrix (eluate A1) and the eluate was bound to the matrix (eluate A2). Furthermore, readings were carried out simultaneously at the maximum wavelength of metNgb (420 nm) and deoxyNgb (415 nm) every 5 minutes for 120 minutes. The graph of the results of the reduction kinetic test for the three samples can be seen in Figure 5 below.

From the three graphs above, it can be seen that there was a decrease in the absorbance ratio of 420nm to 415nm in 25 readings for 120 minutes. It can be seen that the peak eluate of A1 is the isolate that has the best reduction kinetics because it has a linear regression (R2) that is closest to one, which is 0.95.

DISCUSSION

Isolation of the reductase enzyme from bovine brain using a buffered RIPA lysis solution containing a protease inhibitor. The protein yield obtained from salting out with ammonium sulphate was very low, so RIPA buffer was chosen as a reagent for protein extraction from tissue. Furthermore, RIPA buffer is more practical and can be completed in much less time than the salting out technique with ammonium sulphate.

The SDS-PAGE electrophoresis results demonstrated that the isolation and purification processes were successful. 1. Diagram The presence

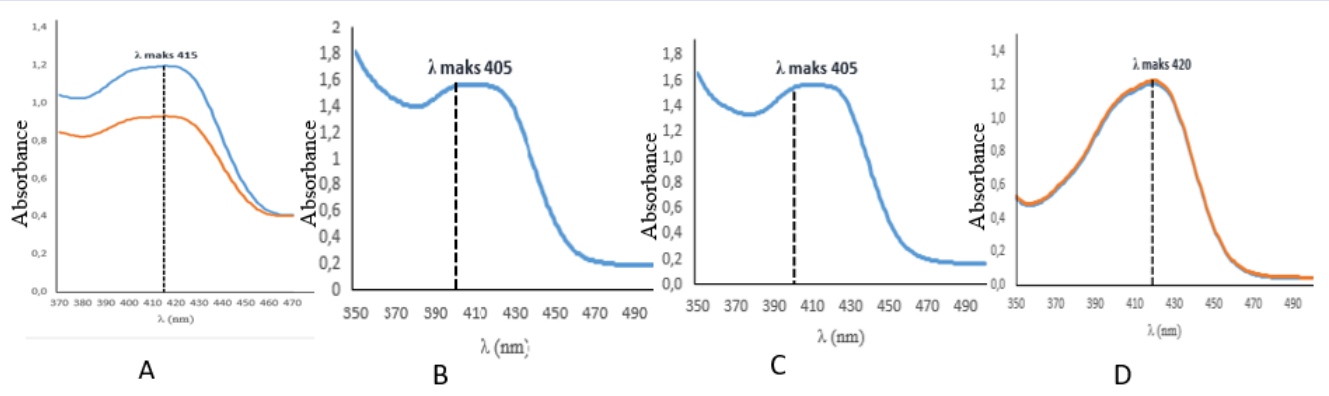

Figure 4: Neuroglobin absorption spectrum, A) purified deoxyNgb spectrum (orange) and recombinant Ngb (blue), B) Purified oxyNgb absorption spectrum, C) Purified carboxyNgb absorption spectrum and D) Purified and recombinant metNgb absorption spectrum.

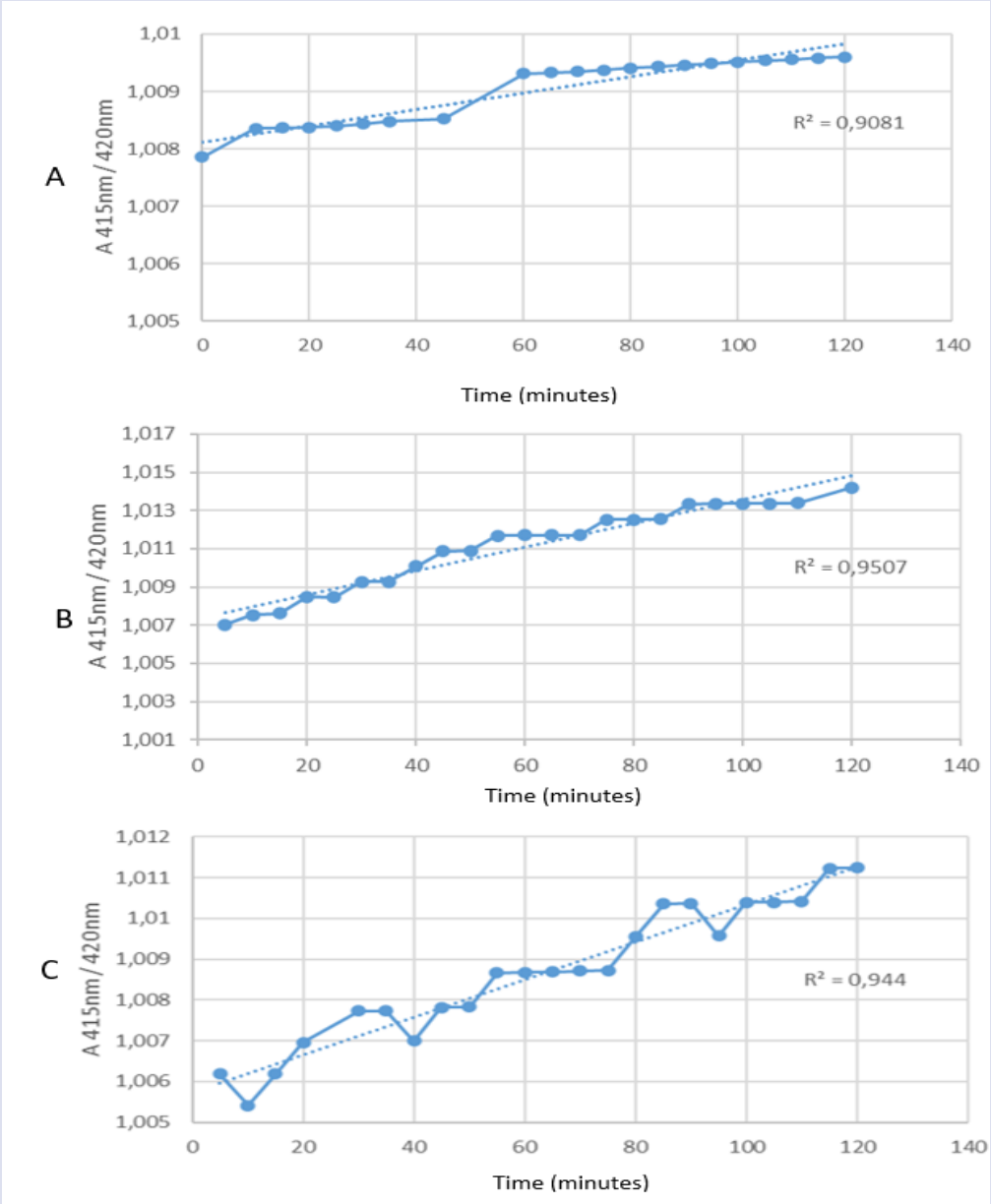

Figure 5: Kinetic reduction of NgbFe^{3+} to NgbFe^{2+}. A) Reduction of NgbFe^{3+} to NgbFe^{2+} using Ngb-free fraction, B) reduction of NgbFe^{3+} to NgbFe^{2+} peak eluate A1 and C) reduction of NgbFe^{3+} to NgbFe^{2+} peak eluate A2.

of a protein with a molecular weight of 17.26 kDa can be seen in the protein bands. A WB confirmation test was also performed to ensure that the protein obtained was Ngb protein. Anti-Ngb antibodies, which can bind specifically to Ngb, are used at this stage. 2nd Figure The analysis revealed that the purified protein was Ngb. The SDS-PAGE electrophoresis results demonstrated that the isolation and purification processes were running smoothly. The protein band, which indicates the presence of a protein with a molecular weight of 72.45 kDa, demonstrates this. The compound is thought to be one that can reduce Ngb-Fe3+ (Figure 3).

Ngb is a heme group-containing protein. The conjugated electron configuration system is responsible for the colour intensity of porphyrins. The absorption spectrum is affected by any change in the symmetry of the porphyrin ring. 8 The maximum absorption peaks differ depending on the Ngb condition. Based on the observations, the maximum absorption peaks of deoxyNgb, oxyNgb, carboxyNgb, and metNgb are 415nm, 405nm, 405nm, and 420nm, respectively (Figures 4.A; 4.B; 4.C and 4.D). Several previous studies have been carried out. According to Dewilde et al. (2001), who used human Ngb generated through recombinant techniques, the maximum absorption peak of deoxyNgb was 426nm. According to Bjrlykke et al. (2012), who used Ngb isolated from salmon, the maximum absorption wavelengths of deoxyNgb, carboxyNgb, and MetNgb are 424nm, 415nm, and 411nm, respectively.

The reduction kinetic test was calculated using the absorbance ratio of deoxyNgb at 415 nm to metNgb at 420 nm (Figure 5). The higher the 415/420 ratio, the more deoxyNgb is formed in solution when compared to metNgb. The 415/420 ratio is plotted against reaction time; the longer the time, the more deoxyNgb is formed, and the relationship between the two parameters is linear.

The free fraction of Ngb, peak eluate A1 and peak eluate A2 were used to reduce the oxidised Ngb. The three samples reduced oxidised Ngb, but peak eluate A1 had the best reducing activity due to the highest regression value. Sahara's previous research discovered that the eluate of the first elution peak had a higher reduction activity than the eluate of the second peak. 11 Meanwhile, Novientri discovered that the eluate of the second elution peak had higher reduction activity than the eluate of the first peak in a study.

According to the research findings, Neuroglobin can be isolated from cow brain tissue. Neuroglobin in bovine brain tissue contains Ngb reductase enzyme analogue compounds that can reduce oxidised Ngb (Ngb-Fe3+) to reduced Ngb (Ngb-Fe2+), as indicated by the maximum absorption of Ngb at 415nm, 405nm, 405nm, and 420nm, respectively, under deoxy, oxy, carboxy, and meth conditions.

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AUTHOR'S CONTRIBUTIONS

Ninik Mudjihartini: Conceptualization, Investigation, Methodology, Validation, Data curation, Resources, Formal analysis, Visualization. Dewi Pratiwi Purba: Conceptualization, Methodology, Formal analysis, Data curation. Fadilah Fadilah: Formal analysis, Writing - original draft, Writing - review & editing, Visualization. Mohammad Sadikin: Conceptualization and Investigation. Sri Widia A. Jusman: Conceptualization, Investigation, Methodology.

DECLARATION OF COMPETING INTEREST

The authors declare "No conflicts of interest".

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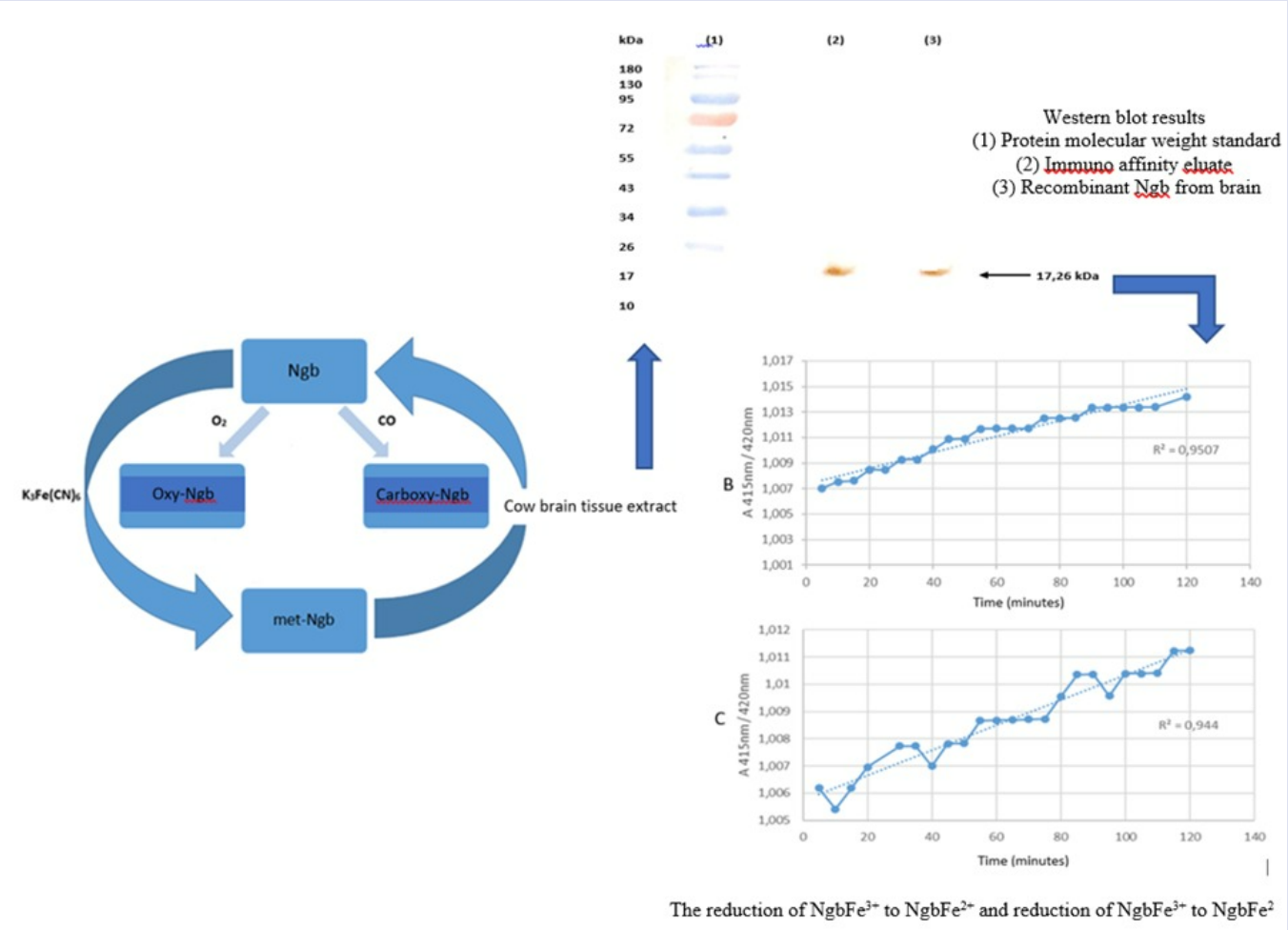
GRAPHICAL ABSTRACT



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