

Antioxidant Biosensor Using Microbe

Dyah Iswantini, Trivadila, Novik Nurhidayat, and Waras Nurcholis

Abstract—The antioxidant compounds are needed for the food, beverages, and pharmaceuticals industry. For this purpose, an appropriate method is required to measure the antioxidant properties in various types of samples. Spectrophotometric method usually used has some weaknesses, including the high price, long sample preparation time, and less sensitivity. Among the alternative methods developed to overcome these weaknesses is antioxidant biosensor based on superoxide dismutase (SOD) enzyme. Therefore, this study was carried out to measure the SOD activity originating from *Deinococcus radiodurans* and to determine its kinetics properties. Carbon paste electrode modified with ferrocene and immobilized SOD exhibited anode and cathode current peak at potential of +400 and +300mv respectively, in both pure SOD and SOD of *D. radiodurans*. This indicated that the current generated was from superoxide catalytic dismutation reaction by SOD. Optimum conditions for SOD activity was at pH 9 and temperature of 27.5°C for *D. radiodurans* SOD, and pH 11 and temperature of 20°C for pure SOD. Dismutation reaction kinetics of superoxide catalyzed by SOD followed the Lineweaver-Burk kinetics with *D. radiodurans* SOD KMapp value was smaller than pure SOD. The result showed that *D. radiodurans* SOD had higher enzyme-substrate affinity and specificity than pure SOD. It concluded that *D. radiodurans* SOD had a great potential as biological recognition component for antioxidant biosensor.

Keywords—Antioxidant biosensor, *Deinococcus radiodurans*, enzyme kinetic, superoxide dismutase (SOD).

I. INTRODUCTION

THE cells in organisms such as “human”, “animal”, and “plant” cells are continuously exposed by many substances which can cause oxidative stress. This conditions can be affected both internally, through normal physiologically process such as mitochondrial respiration, and externally, such as pollutants and irradiations [1]. They cause the formation of reactive oxygen species (ROS), including free radicals which has strong implication with pathophysiological diseases such as cancer, Alzheimers’ disease, etc. The cells are equipped with some defense systems against free radicals, which work through some mechanisms; one of them is antioxidant protection. Some substances knowing to have this

D. Iswantini is with the Department of Chemistry, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Gedung Fapet lantai 4, Jl Agathis, IPB Darmaga, Bogor, Indonesia and Biopharmaca Research Center, Bogor Agricultural University, Bogor, Jl.Taman Kencana no 3 Bogor, Indonesia. (e-mail: dyahprado@yahoo.co.id).

Trivadila is with the Department of Chemistry, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Gedung Fapet lantai 4, Jl Agathis, IPB Darmaga, Bogor, Indonesia and Biopharmaca Research Center, Bogor Agricultural University, Bogor, Jl.Taman Kencana no 3 Bogor, Indonesia. (e-mail: trivadila@gmail.com).

N. Nurhidayat is with Division of Microbiology R&D for Biology, The Indonesian Institute of Sciences, Bogor, Indonesia.

W. Nurcholis is with Biopharmaca Research Center, Bogor Agricultural University, Bogor, Jl.Taman Kencana no 3 Bogor, Indonesia.

protection are ascorbic acid, tocopherol, and also polyphenols which are obtained externally from foods. Besides, various food supplements known also contain antioxidant such as Vitamin C, Vitamin E, and polyphenols.

Antioxidant is needed in so many fields, for example, in human health, food and beverage industries, pharmaceutical industry, and also fuel industry such as biodiesel [2]. Therefore, an appropriate method is needed to measure the antioxidant properties in various types of samples, either sample from natural and industrial products.

Various methods have been developed to measure antioxidant properties, i.e. antioxidant capacity and antioxidant activity. Common techniques are spectrophotometry, fluorescent, liquid or gas chromatography, etc. These traditional methods, especially spectrophotometry, have some disadvantages, i.e. expensive cost because using many kinds of reagents in large amounts, long time analysis because of long procedure sample preparations, and less sensitivity specially in measuring colorful and turbid sample. Therefore, an appropriate, easy, rapid, and sensitive method is needed to study the antioxidant properties.

Electrochemical biosensor is an alternative method developed to study the antioxidant properties. Electrochemical antioxidant biosensor is a promising tool, suitable for rapid analysis, inexpensive-instrument, and simple operation protocol. There are two kinds of antioxidant biosensors, first, amperometric biosensor to detect monophenol and polyphenol antioxidant (the main antioxidant in foods). It was tyrosinase, laccase, or peroxidase based biosensor [3]. Second, biosensor to measure the antioxidant capacity based on free radical scavenging. To measure the antioxidant capacity based on measurement of superoxide radical ($O_2^{\bullet-}$) concentration, two types biosensor have been developed, using cytochrome c (cyt c) or superoxide dismutase (SOD) enzyme. Superoxide radical determination using cyt c-based sensor is less selective because the heme protein was not specific to $O_2^{\bullet-}$. SOD-based biosensor is more specific and sensitive. Another method to determine antioxidant capacity is by measuring the free radical-caused DNA damage. In this method, the presence of antioxidant involves decreasing of DNA alteration [4].

Enzyme-based biosensor consists of an enzyme as component of biological recognition and a transducer. Transducer can be an amperometry, potentiometry, conductometry, optic, or calorimetry transducer. Electrochemical enzyme-based biosensor has been applied to detect various substrates which are selectively oxidized or reduced in catalytical process. SOD-based biosensor to measure the antioxidant capacity has shown more promising performance compared to cytochrome c-based biosensor [4]. SOD-based biosensor has been shown to measure antioxidant

capacity in various kinds of samples such as natural products [5]-[7], food and beverage products [7]-[9], and pharmaceutical products [10]-[12], either in water-based or organic solvent-based sample [13]. This method also showed to have better sensitivity compared to spectrophotometry and spectrofluorometry methods [8], [11]-[12], [14].

SOD originated by various microbes, such as *Aspergillus niger*, *Saccharomyces cerevisiae*, *Escherichia coli*, and so on [15]-[17]. One of enzyme-producing bacteria in Indonesia is *Deinococcus radiodurans*. *D. radiodurans* is gram positive, anaerobe, non-pathogen bacteria which very resistant to UV radiation, ionization, desiccation, and ROS. UV radiation itself can produce radicals which can harm the bacteria cells. It is natural to expect that *D. radiodurans* is very resistant to the extreme environment because *D. radiodurans* has high activity SOD. Specific activity of Mn-SOD produced by *D. radiodurans* was 1250Umg⁻¹, measured by colorimetric method [18]. Mn-SOD generated by *Deinococcus* bacteria was an enzyme that can be induced to increase its activity. Potassium superoxide addition and UV radiation could increase the activity of generated Mn-SOD [19]. Mn-SOD which was reconstituted from its apoenzyme could reach 3300Umg⁻¹ [20]. This value was higher compared to Mn-SOD form thermophilic fungi *Chaetomium thermophilum*, i.e. 115.77Umg⁻¹ [21], and almost the same with specific activity of Mn-SOD from bovine erythrocyte which was produced commercially [22]. Based on this, SOD originated from *D. radiodurans* has potency to utilize as biological recognizer in antioxidant biosensor. However, there is no report about immobilized *D. radiodurans* SOD activity which is measured electrochemically. Therefore, this study was carried out to investigate the activity of *D. radiodurans* SOD which was immobilized onto carbon paste electrode surface and to determine its kinetic properties using electrochemical method. This was interesting because all this time SOD used in antioxidant biosensor was SOD isolated from bovine erythrocyte.

II. MATERIALS AND METHODS

A. Microbial Cells and Chemicals

D. radiodurans were grown on heterotrophic media contained trypton 1%, yeast extract 0.5%, glucose 0.2%, and NaCl 0.5% for 2 hours at 300°C to a late logarithmic phase. They were then incubated to reach the OD₆₁₀ of 0.5. The cells harvested by centrifugation were washed twice with a saline solution (0.85 % NaCl) and were kept at 5°C.

Q₀ (2,3-dimethoxy-5-methyl-1,4-benzoquinone), ferrocene (Fe(C₅H₅)₂), and superoxide dismutase (SOD) are from Sigma Chemical Co. All other chemicals used were of high purity.

B. Preparation of the SOD Modified Electrode [23]-[24]

A carbon paste was constructed by packing a mixture of graphite powder, mediator and paraffin liquid with graphite-paraffin ratio 2:1 and mediator concentration was 1mg mediator/100mg graphite into one end of a glass tubing, and

the surface was smoothed using a piece of waxed paper. Onto the surface of carbon paste electrode, a 10µl of aliquot of the SOD crude extract from *D. radiodurans* was dropped on to the surface. The solvent was allowed to evaporate. Next, the surface was covered with a dialysis membrane, and fixed with nylon fibre.

C. Electrochemical Measurement

Electrochemical measurements were carried out with eDAQ potentiostat under anaerobic conditions, in which an Ag|AgCl|Sat. KCl electrode, a platinum disk, and carbon paste electrode were used as reference electrode, counter electrode, and working electrode, respectively. The measurements were conducted at room certain temperature with an electrolysis cell containing 1mL of basal solution of phosphate buffer at a certain pH. The optimum temperature and pH were previously determined. The test solution was stirred with a magnetic stirrer and was deaerated by passing over nitrogen gas, unless stated otherwise.

D. Kinetics Properties Determination

Kinetic properties determination was done after the optimum conditions for immobilized SOD activity (pH, temperature, and the mediator) were obtained. The general procedure was the same, but in kinetic assay, superoxide radical concentration (substrate) concentration was varied between 0.00-1.00mmol L⁻¹ of xanthine concentration.

The kinetics properties of immobilized *D. radiourans* SOD extract was determined using Michaelis-Menten equation:

$$I = \frac{I_m^{app} [xantina]}{K_m^{app} + [xantina]}$$

with I_m^{app} was measured maximum current response (apparent), K_m^{app} was apparent Michaelis-Menten constant, and [xantina] was xanthine concentration.

Next, from Michaelis-Menten equation derivation, Lineweaver-Burk dan Eadie-Hofstee plots were made.

III. RESULTS AND DISCUSSION

A. D. radiodurans Cell Growth and SOD Extraction

D. radiodurans cells were grown in heterotrophic media with and without 8% ethanol addition. Ethanol was added to give extreme condition for growth environment of *D. radiodurans*. It was expected that *D. radiodurans* would grow rapidly and abundantly. Next, the growth rate and *D. radiodurans* cells quantity in with and without ethanol media were compared. *D. radiodurans* growth curve was shown in Fig. 1.

Fig. 1 shows that ethanol addition could increase growth rate of *D. radiodurans* cell. The optical density (OD) values were also higher than its values when cells were growth in original heterotrophic medium. Generation rate in medium with ethanol was 13 folds higher than in original medium, i.e. 0.0177 per hour in medium with ethanol and 0.0014 in medium without ethanol.

D. radiodurans is among bacteria that can endure the extreme environments such as UV radiation, heating, desiccation, dehydration, acid condition, and alcohol treatment. *D. radiodurans* was known to have *irrE* exogene. *irrE* exogene was a regulator protein that is responsible to regulation of *recA* and *pprA* genes expression which were responsible to *D. radiodurans* resistances to extreme environment, especially gamma (γ) irradiation [25]. *D. radiodurans irrE* gene expressed in *Zymomonas mobilis* showed to make *Z. mobilis* more resistant to acid and ethanol [26].

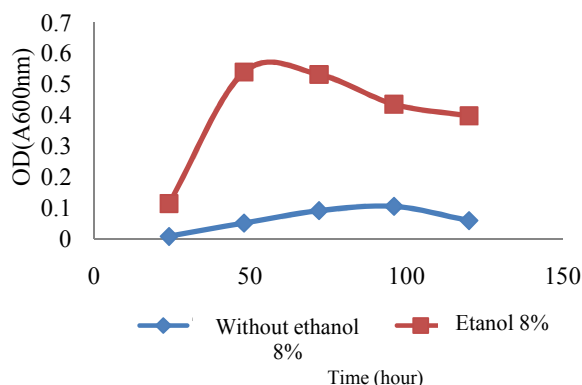


Fig. 1 *D. radiodurans* growth curve in heterotrophic medium

Besides increasing growth rate and cell quantity of *D. radiodurans*, ethanol addition was also expected to increase Mn-SOD activity which was produced by *D. radiodurans*. It was successfully in the fellow genus *Deinococcus*, *D. radiophilus*, which also had endurance to extreme conditions. *D. radiophilus* was also produced Mn-SOD. With potassium superoxide, *D. radiophilus* growth increased up to 60% and SOD band intensity dense in protein stain also increase up to 15%. It supported that Mn-SOD produced by *D. radiophilus* was an enzyme which would increase qualitatively and quantitatively along with the increasing of environmental pressure [19]. It explained that Mn-SOD production in *Deinococcus* cells and its activity could be induced by giving extreme condition in its growth environment such as ethanol or acid addition, UV or γ radiation, etc.

After it was harvested, cells wall was lysed to extract its cytoplasm protein containing SOD. Extracted protein has concentration $701.02 \mu\text{g mL}^{-1}$. Protein extract yield was 0.14%. It was smaller than Fe-SOD extract from *Pseudomonas ovalis*, i.e. up to 0.95% [27] or Cu/Zn-SOD from *E. coli* which reaches 1.24%. The small value of *D. radiodurans* Mn-SOD probably because *D. radiodurans* has thicker and stronger cell wall than common bacteria and yeast cells. *D. radiodurans* contains thick peptidoglycan and outer membrane outside its cell wall [28]. Besides, *D. radiodurans* cells were in tetrad-shaped and bigger in size so that it was difficult to break the cells and extract its cytoplasm.

Reference [20] purified Mn-SOD from *D. radiodurans*. Their results showed that the method used could produce pure Mn-SOD with protein content up to 45% from its cytoplasm

protein extract. From their research, also known that Mn metal in active sites of Mn-SOD could be removed or replaced reversibly. Apo-Mn-SOD could be obtained with protein extract dialysis using guanidium chloride and 8-hydroxyquinoline. Apo-Mn-SOD could be reconverted into holo-Mn-SOD by MnCl_2 addition. Based on [29], apo-glucose dehydrogenase (apo-GDH) produced by *E. coli* could be changed into holo-GDH by titration using pyrrolo quinone quinone (PQQ) as its prosthetic group, so that the amount of GDH produced by single cell of *E. coli* could be determined with electrochemical method. With the same way to *D. radiodurans*, it was expected that Mn-SOD produced by single cell of *D. radiodurans* could be determine by titrating of apo-Mn-SOD with MnCl_2 into holo-Mn-SOD and its activity could be measured using electrochemical method.

B. Optimization of Immobilized *D. radiodurans* SOD Activity Measurement

Measurement of immobilized *D. radiodurans* SOD activity was carried out using response surface as experimental design method. Briefly, the parameters optimized were pH (7-11), temperature (20-35°C), and [ferrocene] (1-5mg/100mg graphite).

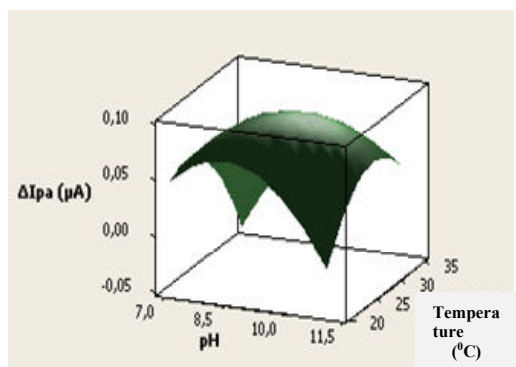
Fig. 2 shows surface plot of pH and temperature to oxidation current peak (a), pH and mediator to oxidation current peak (b), and temperature and mediator to oxidation current peak (c). Those plots showed optimum activity of immobilized SOD, signed with maximum peaks.

Figs. 2 (a) and (b) show the influence of pH to the immobilized SOD activity. Immobilized SOD activity was optimum in pH range 8-10, while from Figs. 2 (b) and (c), it is seen that optimum activity of immobilized SOD was mediator concentration between 2-4mg/100mg graphite. And also, from Figs. 2 (a) and (c), temperature optimum for immobilized SOD activity is between 22-30°C. So it was concluded that optimum condition for immobilized *D. radiodurans* SOD activity was at pH 9, temperature 27.5°C, and ferrocene concentration 3 mg/100 mg graphite.

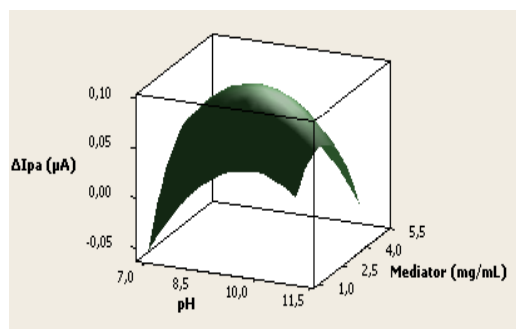
This result was different with other studies. Reference [30] immobilized SOD with ferrocene as mediator and carboxyaldehyde cross-linked with glutaraldehyde on Pt electrode surface, and gained optimum activity at pH 7.4 and 37°C. While [13] immobilized SOD onto oxygen electrode between dialysis and cellulose triacetate membranes at pH 10.2 and 20°C. SOD immobilized within kappa-carrageenan membrane had optimum activity at pH 7 and 20°C.

C. Optimization of Immobilized SOD Activity Measurement

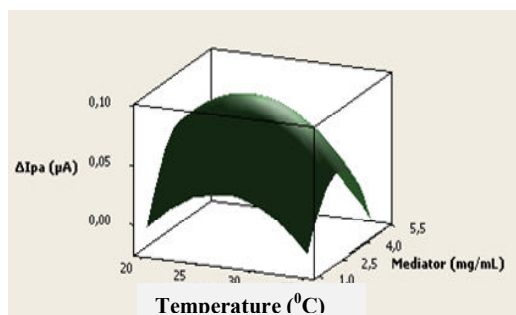
Optimum condition of immobilized pure SOD activity was different with immobilized *D. radiodurans* SOD activity. Optimum pH and temperature of immobilized *D. radiodurans* SOD activity were in the middle range of optimized parameter values and had maximum peaks, while optimum pH and temperature of immobilize pure SOD activity were in the side range of optimized parameter values and had minimum peaks (Fig. 3). Optimum condition for immobilized pure SOD activity was at pH 11 and temperature 20°C.



(a)



(b)



(c)

Fig. 2 Surface plot of (a) pH and temperature to oxidation current peak (b) pH and mediator to oxidation current peak and (c) temperature and mediator to oxidation current peak

The difference of optimum condition could be affected by the purity level of protein extract itself, and also by purification method applied. *D. radiodurans* SOD contained protein extract still had other various proteins beside the SOD enzyme protein. With the existence of other proteins, it was suspected that various interactions between those proteins would affect the ionic strength and net pI value, which in the end, would shift the optimum condition. *D. radiodurans* had optimum temperature growth at 30°C [28], so that its activity would be optimum around this optimum temperature growth.

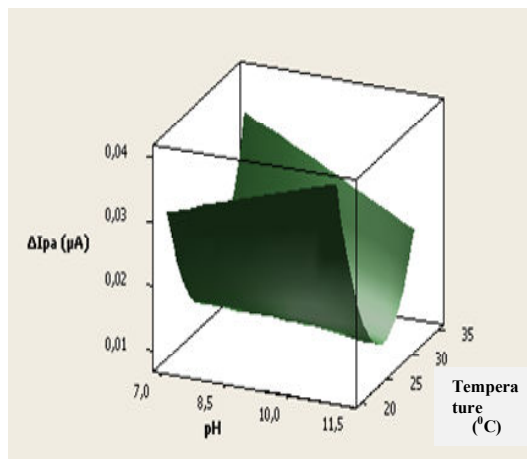


Fig. 3 Surface plot of pH and temperature on immobilized SOD activity

D. Kinetics Properties of Immobilized SOD

Fig. 4 shows linear relationship between xanthine concentration and immobilized SOD and *D. radiodurans* SOD activity. As could be seen, immobilized pure SOD activity had broader linear range than immobilized *D. radiodurans* SOD activity, i.e. between 0.10-0.70mM for immobilized pure SOD and 0.10-0.60mM for immobilized *D. radiodurans* SOD. However, the R² value of immobilized pure SOD was lower than immobilized *D. radiodurans* SOD. Besides, it could also be seen that immobilized *D. radiodurans* SOD had higher activity than immobilized pure SOD. Although it still needs other parameters analysis, but this result could show that *D. radiodurans* SOD immobilized onto carbon paste electrode had potency to give more accurate measurement than pure SOD.

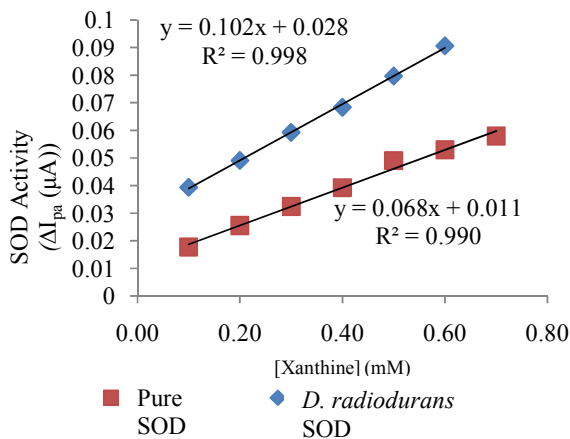


Fig. 4 Linearity of xanthine concentration and immobilized SOD activity

Linear ranges obtained from this study were still narrower than other studies. Reference [10] immobilized SOD in Kappa-carragenan membrane got linear range between 0.02-2.00mM. Reference [31] developed third generation biosensor

by immobilized SOD within sol-gel thin layer onto gold electrode surface produced more sensitive response with linear range between 0.2-1.6 $\mu\text{mol L}^{-1}$ of superoxide radical. While [30] made antioxidant biosensor which could measure within linear range of 0-100 μM of hypoxanthine concentration.

To study the specificity of *D. radiodurans* SOD immobilized onto carbon paste electrode, we performed enzymatic kinetic properties determination for this enzyme,

i.e. apparent Michaelis-Menten constant (K_{Mapp}) and apparent maximum rate ($V_{maks\ app}$) which was analogous with apparent maximum current ($I_{maks\ app}$). Those kinetic properties were determined with two methods or approaches, Lineweaver-Burk and Eadie-Hofstee. Figs. 5 and 6 show Lineweaver-Burk curve, relationship between $1/[\text{xantina}]$ and $1/\Delta I_{ap}$, and Eadie-Hofstee curve, relationship between ΔI_{ap} and $\Delta I_{ap}/[\text{xantina}]$. K_{Mapp} and $I_{maks\ app}$ values were given in Table I.

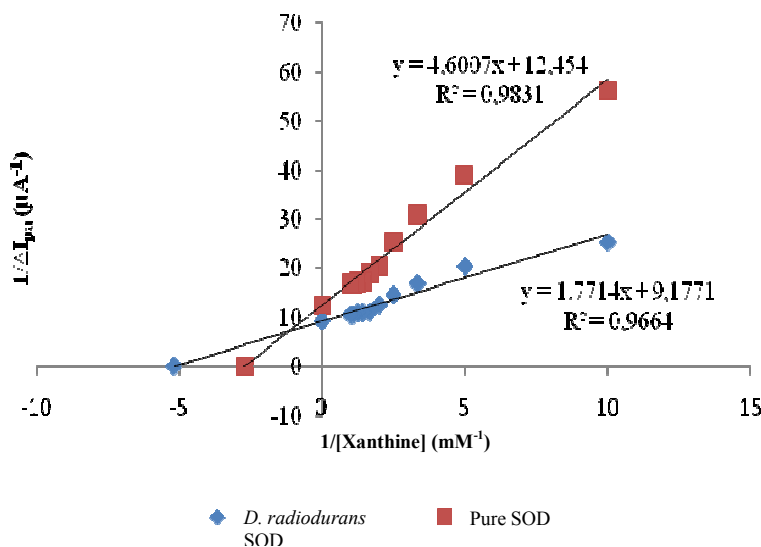


Fig. 5 Lineweaver-Burk plot of immobilized SOD

$K_{M\ app}$ and $I_{maks\ app}$ values obtained between Lineweaver-Burk and Eadie-Hofstee methods were different (Table I) both in pure SOD or *D. radiodurans* SOD. This was because the linearity obtained in both methods did not reach 0.9999 (Figs. 5 and 6). However, linearity of Lineweaver-Burk method was higher than Eadie-Hofstee method. So it was concluded that enzymatic reaction kinetics of superoxide dismutase catalyzed by immobilized SOD followed the Lineweaver-Burk kinetic plot.

By considering that immobilized enzyme was not disturbed by its substrate, especially diffusion flow, then, from Table I, it could be seen that immobilized *D. radiodurans* had higher $I_{maks\ app}$ value than immobilized pure SOD for both methods. This showed that catalytic reaction rate of superoxide dismutase to O_2 and H_2O_2 catalyzed by immobilized *D. radiodurans* was faster than immobilized pure SOD. Besides, K_{Mapp} values of immobilized *D. radiodurans* SOD was smaller than immobilized pure SOD in Lineweaver-Burk method, showed that the affinity of immobilized *D. radiodurans* SOD to superoxide radicals was higher than immobilized pure SOD. It probably was caused by the difference in structure. It is concluded that *D. radiodurans* SOD had a great potency as biological recognition component for antioxidant biosensor. The result was supported by previous result that indicated that SOD biosensor could be used for in situ determination of antioxidant capacity of all the different samples of bread and flour tested, other advantages of this method were simple,

cheap, and could be used for numerous analyses [32].

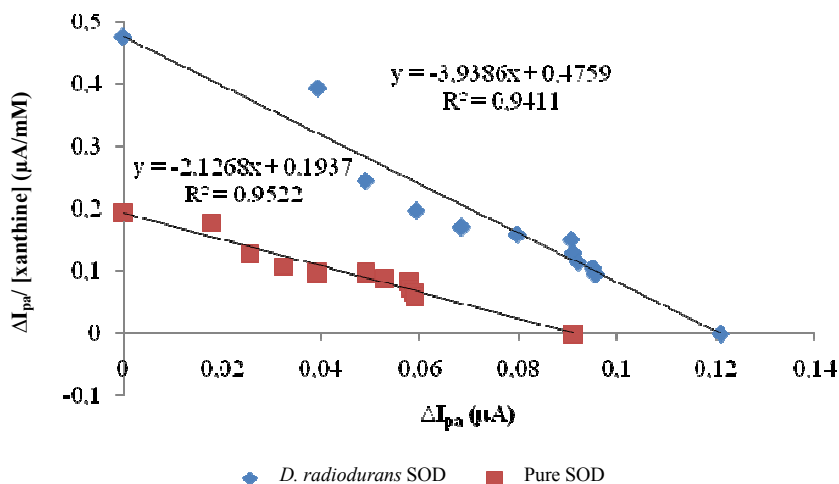


Fig. 6 Eadie-Hofstee plot of immobilized SOD

TABLE I
KINETICS PROPERTIES VALUES OF IMMOBILIZED SOD

Method	Pure SOD		<i>D. radiodurans</i> SOD	
	$I_{maks\ app}$ (μA)	K_{Mapp} (mM)	$I_{maks\ app}$ (μA)	K_{Mapp} (mM)
Lineweaver-Burk	0.0803	0.3694	0.1089	0.1930
Eadie-Hofstee	0.1937	2.1268	0.4759	3.9385

Pure SOD was isolated from bovine erythrocyte which had Cu/Zn-SOD type [22], while *D. radiodurans* SOD was Mn-SOD type [18]. Cu/Zn-SOD and Mn-SOD had different structure. Cu/Zn-SOD is a dimer enzyme with every monomer contained one Cu active site and one Zn active site connected by imidazole histidine. Cu was binded to other three histidines and formed distorted planar square structure with one addition of water molecule. Zn was binded to two histidines and one aspartate as an addition at imidazole bridge (Fig. 7 (a)). Cu was redox-active metal, it changed its oxidation state between 2+/3+ during catalysis process, and Zn had a role to keep the stability of enzyme structure. Mn-SOD was a dimer enzyme with one Mn atom for every unit. At active site, Mn was binded to three histidine residues and one aspartate acid residue and solvent molecule as fifth ligand. This solvent ligand was postulated as hydroxide when Mn in oxidation state and for protonation process during Mn reduction (Fig. 7 (b)).

Review about kinetic study by [33] explained that Cu/Zn-SOD and Mn-SOD had different mechanism in dismutation reaction. Radio pulse study showed that catalytic reaction of Cu/Zn-SOD was diffusion controlled and did not depend on pH at pH range 5-9.5 with k_{cat} value $k_{cat} \approx 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$. This catalytic process was followed the reaction:

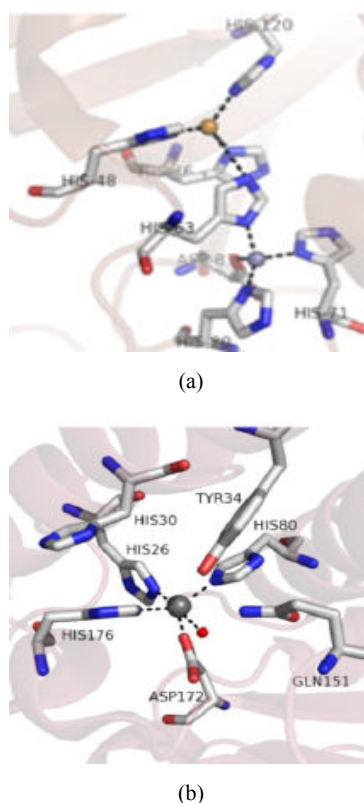
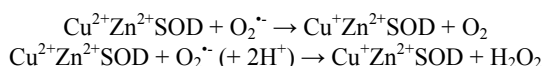
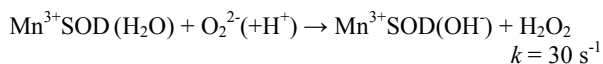
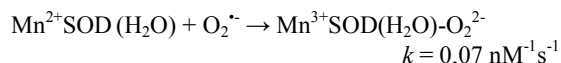
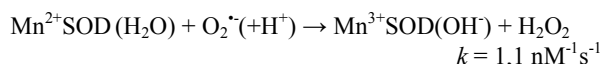
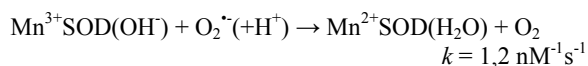


Fig. 7 Cu/Zn-SOD structure (a) and Mn-SOD *D. radiodurans* structure (b) [33]

While, dismutation reaction mechanism in Mn-SOD followed “explosion” phase and “zero order” phase. These mechanisms allowed reduced state of Mn-SOD would react with superoxide via two pathways. Overall proposed reaction mechanisms, including inhibition complex formation Mn3+SOD-O22-, followed below reactions [34]:



Cu/Zn-SOD had higher rate constant than Mn-SOD. Catalytic reaction of superoxide dismutation by Cu/Zn-SOD was very fast and diffusion controlled [35], while catalytic reaction by Mn-SOD was not diffusion controlled. Immobilization process performed was probably could decrease diffusion rate so that enzymatic reaction rate by immobilized Cu/Zn-SOD also decrease.

As enzyme was used as recognition component in biosensor, one factor that should be considered was analyte diffusion into sensor. In this condition, diffusion or relatively dominant reaction would affect biosensor performance. Some factors which affected substrate and product concentration into and within sensor were external membrane permeability, substrate diffusion and product reaction in detector. If reaction rate was limited by external membrane, diffusion transport within enzyme area was faster than when through external membrane. This could be happen when enzyme area was thinner than membrane thickness and analyte diffusion was better in enzyme matrix than membrane material. If reactant diffusion at enzyme area was lower than membrane matrix material, then substrate and product concentration gradient within membrane were ignored. Analyte diffusion rate to reach active site could also be affected by the position or orientation of enzyme active site when enzyme was immobilized. Immobilization method could also determined this orientation. If immobilization method performed did not result in active site changes, so it was expected that there was no any substrate rate hindrance into active site at enzyme matrix. Besides, external membrane selection which was not too thick was also expected to decrease membrane hindrance against substrate rate toward enzyme matrix.

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