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Immobilization of Cholesterol Oxidase from *Streptomyces* Sp. on Magnetite Silicon Dioxide by Crosslinking Method for Cholesterol Oxidation

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Abstract
Enzymatic biosensor has been paid much attention to the research fields due to its advantage in medical application. As one of the application, we determined the optimum value of cholesterol oxidase against cholesterol. In this work, we studied the behavior of cholesterol oxidation by enzymatic reaction to get the optimum condition for cholesterol oxidation. The enzyme that used were in two form, free cholesterol oxidase, and immobilized cholesterol oxidase. Cholesterol oxidase was produced from *Streptomyces* sp. by using solid state fermentation method and identified had high enzyme activity to be 5.12 U/mL. Cholesterol oxidase was simultaneously crosslinked immobilized onto magnetite coated by silicon dioxide (M-SiO_2). The support was characterized by Fourier transform infrared (FTIR) to determine the functional group of modified particle and scanning electron microscope (SEM) to observe the morphological or our prepared particle. Cholesterol oxidase sensitivity to substrate was analyzed by using HPLC with different interval time measurements. The oxidation of cholesterol by free enzyme and immobilized enzyme was also investigated. The best sensitivity of cholesterol oxidase was estimated to oxidize Cso (concentration of substrate) 1.46 mM of substrate with Ce (concentration of enzyme) 20 mg/mL for 180 min. Final oxidation value of cholesterol by immobilized enzyme was greater than 60%. The results of this study revealed that immobilized enzyme for cholesterol oxidation was stable, reproducible, and sensitive.

Keywords Biosensor · Cholesterol · Cholesterol oxidase · Enzymatic · Magnetite · Oxidation

Introduction

Cholesterol and fatty acid are important sources in human body as biological membranes to form nerves and brain cells. Cholesterol and its protein are called lipoprotein. The lipoprotein

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is classified into two forms, low density lipoprotein (LDL), and high density lipoprotein (HDL). A high level of LDL could increase the risk of health such as blood coagulation, arteriosclerosis, heart attack, stroke, and heart coroner [1].

Regarding to the importance of monitoring the health issue, cholesterol level is appealed to be monitored annually by quantification of blood cholesterol level. There was a method called cholesterol quantification by single strip. This method needs some microliters of blood to be injected into the strip. The disadvantage of this method is the single use of strip test which is non-economic friendly. Numerous methods for cholesterol detection have been well developed such as disposable electrochemical sensor [2], flexible micro-needle electrode array [3], ratiometric fluorescence sensor on enzyme [4]. These methods required an optimum amount of cholesterol oxidase to identify the cholesterol.

Nowadays, biosensor, is a new and advance method to develop the quantification of cholesterol level. There were many modifications to develop the performance of biosensor, such as compositing materials, fluorescence biosensor, chip modification to the surface, and combining three enzymes [5, 6].

However, there is no optimum substrate or enzyme concentration for detecting the target molecules. Hereby, in this work, we studied the effect of substrate and enzyme concentration to determine the optimum condition for quantifying the cholesterol. Determination of cholesterol by an enzymatic method generally involves two enzymes, cholesterol oxidase and cholesterol esterase. This method has great specificity and sensitivity. The use of cholesterol oxidase is more common rather than cholesterol esterase due to its direct reaction against the substrate [7].

Cholesterol oxidase (ChOx) is an enzyme which catalyzes the oxidation of cholesterol and converts 5-cholesten-3β-ol into 4-cholesten-3-ones. ChOx receives considerable attention around the world due to its use in enzymatic cholesterol analysis, especially biosensor as a new advance method. ChOx is isolated from a variety of organism sources such as fungi and bacteria. This enzyme can be produced by a bacterium in three forms: intracellular, extracellular, and membrane bound. Numerous researches have proofed that the catalyzer in the first step of the cholesterol degradation was cholesterol oxidase [8].

Moreover, ChOx is useful for biotechnological implementation as well as industrial implementation, for example determination of cholesterol serum in blood, insecticides, larvicides, and antibiotic for pathogen bacteria *Rhodococcus equi* [8]. ChOx is used as a biocatalyst that provides valuable steroidal compounds and non-steroidal chiral compounds. ChOx from *Chromobacterium sp. DS-1* can be used to reduce oxysterol cytotoxicity [9]. The methods for producing enzyme are a crucial thing that needs to be considered. Solid state fermentation is a method to produce an enzyme by using biomass as a substrate with liquid fermentation. In this study, ChOx is produced from *Streptomyces* sp. with solid state submerged fermentation.

In order to enhance the performance of ChOx, it is needed to add supports to the enzyme. There are several types of support which are used to immobilized with the enzyme such as sepharose polymer [10], carbon types from grapheme [11], and carbon nanotube [12], metals like magnetite [5, 13] or gold nanoparticles [14]. These supports can exhibit enzyme and increase the physical or chemical properties as well as the target.

The use of metal in enzyme immobilization became an interesting topic, and the addition of support in enzyme has been performed in many ways of methods. Nevertheless, it is needed to observe the specific support for oxidation to obtain the optimum value. The novelty of this research is the use of support with metal–metal form in cholesterol oxidase for cholesterol oxidation in various concentrations. Magnetite is known as a semiconductor metal with wide

application in medicine or electrochemical application [5, 13]. Hence, the support is used to immobilize the enzyme; it is needed to study and considered about the reaction between support and enzyme. In this work, magnetite was synthesized and coated with silicon dioxide materials. Silicon dioxide acts as a coater and a gap agent between enzyme and magnetite. Silicon dioxide surface hypothesized has a potential application as a bridging of chemical so that it will not distract the targeted compounds directly.

All the compounds interact and revealed to be in oxidation reaction. The oxidation reaction will be conducted with HPLC instrument. The oxidation reaction is established different substrates and enzyme concentrations by the time reactions. Therefore, the aims of this research are to determine the degradation of cholesterol and obtain the final concentration value of substrate after oxidation reaction. At last, this study could be used as reference for maximizing the use of cholesterol oxidase in industry.

Materials and Methods

Materials and Reagents

All chemicals used for enzyme production and metal synthesis originated from analytical reagent grade and used without further purification. An isolate of *Streptomyces* sp. was purchased from LIPI (Indonesian Institute of Science). The chemicals used for bacterial preculture were purchased from Sigma-Aldrich, such as $CaCO_3$, malt extract, glucose agar, NH_4NO_3, K_2HPO_4, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, NaCl, Tween 80 and yeast extract. For submerged fermentation, the addition chemicals from Sigma Aldrich were cholesterol, pepton, NH_4Cl, Na_2HPO_4, $ZnSO_4$, and $CaCl_2$.

Synthesizing of magnetite silicon dioxide used analytical reagent grade chemicals which were purchased from Merck. The chemicals are NH_4NO_3, (3-aminopropyl) triethoxysilane (APTES), NH_4SO_4, Fe_3O_4, tetraothosilicate (TEOS), ethanol, and glutaraldehyde (GA). Aqua demineralization was also used for washing materials to avoid the contaminant from other minerals.

Apparatus

Oxidation measurement was carried out by high performance liquid chromatography (HPLC) with UV-Vis detector and Betasil column 150 mm × 4.6 mm with reversed phase C-18 packing. Injector is model 7725 Rheodyne injector with 10-μl sample loop. The detector model is 484 tunable UV with Perkin-Elmer series 410 pump. The eluents are methanol: isopropanol (70:30) (v/v). The oxidation experiments were measured at 37 °C. Fourier transform infrared (FTIR) and scanning electron microscope (SEM) were employed for studying the functional group and morphology of our synthesized material.

Procedures

Enzyme Production

The production method of crude cholesterol oxidase extract was obtained through submerged fermentation by the bacterium *Streptomyces* sp. Bacterial sub-cultures were carried out

periodically every 2 weeks with experimental stages consisting of the preparation of culture media, bacterial inoculation, and bacterial incubation. Submerged fermentation stages by *Streptomyces* sp. consisted of preparations for pre-culture media, bacterial inoculation, and incubation. The fermentation stage had many similarities to the pre-culture stage.

A crude extract of the cholesterol oxidase enzyme was obtained by the separation method with centrifugation 10.000 rpm for 10 min. The supernatant then filtered using Whatman No.1 filter paper to obtain a crude extract of the cholesterol oxidase. The crude extract was stored at 4 °C before it is used to prevent enzyme inactivation and the enzyme preserved with freeze-drying techniques.

Synthesis of Magnetite Modified Silicon Dioxide (M-SiO_2)

The cholesterol oxidase enzyme was immobilized with double metal materials. The synthesis of magnetite silicon dioxide particles was carried out by coating the magnetite with silicon dioxide precursor. The synthesis of magnetite particles was carried out based on the modification of the method conducted by Huang (2015) [5].

Magnetite silicon dioxide material was synthesized by using the modified sol-gel method. The activated magnetite powder allowed the interaction with the mixtures of 155 mL ethanol, 72 mL aqua demineralization, and 0.5 g NH_4NO_3. The mixture was sonicated for 10 min then stirred rapidly for 6 h at ambient temperature and add slowly 1.5 mL of TEOS into the mixture. The product then washed with aqua demineralization and dried at 60 °C.

The particles were coated with one layer silicon dioxide and then the modified magnetite coated again with silicon dioxide by following the reaction step. The powder products were interacted with 0.17 g NaCl, 0.15 g NH4SO4, and 25 mL ethanol into 50 mL aqua demineralization. The mixture was sonicated for 10 min then add drop by drop 1.5 mL TEOS into the mixture. The reaction was stirred for 8 h at 25 °C. The final product was characterized by using FTIR and SEM.

Immobilization of Cholesterol Oxidase on Magnetite Silicon Dioxide

The immobilization method was carried out by adding 30 mg of magnetite particles into 8 mL of 2.5% (v/v) APTES solution with 25 mL ethanol and stirred at 25 °C for 6 h. After the gel was formed, the solvent was evaporated with boat porcelain in oven at 100 °C for 30 min. The modified particles were then washed with H_2O and ridged in 8 mL phosphate buffer solution (PBS 0.01 mol/L, pH = 7). Next, the addition of 1 mL 25% GA (v/v) and stirred well with a water shaker bath for 90 min. The mixture was then washed several times using a 0.1 M pH 7 phosphate buffer. After that, centrifugation was carried out to separate the particles.

Immobilization of the cholesterol oxidase enzyme with magnetite particles was carried out by adding 10 mL of the cholesterol oxidase enzyme with a mixture of activated magnetite particles into the tube and reacted for 12 h at 4 °C. The resulting product was stored in phosphate buffer solution (0.01 M, pH = 7).

The enzyme loading rate was calculated using a general equation for immobilization:

$$\text{Enzyme loading rate}\ (\%) = \frac{(C_a - C_b)V_s}{W_s} \quad (1)$$

where C_a and C_b refers to concentration of the enzyme initial and supernatant solution after immobilization (mg/mL), respectively. V_s is the volume of the reaction (mL), and W_s is the weight of magnetite silicon dioxide (mg) used for immobilization in the present study.

Oxidation of Cholesterol

The aims of oxidation test were to determine the concentration of cholesterol oxidized by the cholesterol oxidase. The samples tested were crude extracts of the cholesterol oxidase enzyme, and the cholesterol oxidase enzyme immobilized by the silica magnetite material. The oxidation test was carried out using an HPLC instrument with the mobile phase of methanol: propanol (70:30) (v/v).

Results and Discussion

Production of Crude Cholesterol Oxidase

Cholesterol oxidase has been well developed for sensing or biosensing. In the present study, cholesterol oxidase isolated from *Streptomyces* sp. strain. The crude cholesterol oxidase extract was produced by the submerged fermentation method. Liquid medium was used as a growth medium for microorganisms. The crude enzyme extract was produced from a culture filtrate process with an activity of 5.12 U/mL. Meanwhile, the previous work revealed that *Streptomyces* sp. strain had high enzyme activity loaded up to 99 U/mg [15]. *Streptomyces sp.* is one of the organisms that produce cholesterol oxidase enzyme. The crude extract of the cholesterol oxidase was produced after freeze drying process and amounted to 72.05 g with an initial volume of extract of 350 mL. The crude cholesterol oxidase powder was prepared with various enzyme concentration, 5, 10, and 20 mg/mL with the solvent in the form of a phosphate buffer with a concentration of 0.1 M pH 7.0. The prepared enzyme was used for immobilization process with exact amount.

Magnetite Silicon Dioxide Characterization

The magnetite particles (Fe_3O_4) are black fine powder. Magnetite has the ability to bind other metal or magnetic attraction. The process of synthesis of magnetite coated with silica was carried out by mixing conditions through an ultrasonic instrument. The ultrasonic process is allowed the magnetite powder completely dispersed in anhydrous ethanol solution [14]. The use of magnetite as a support in several enzymes has been revealed. Magnetite has a good physical characteristic for thermal stability. Somehow, enzyme is well known as a sensitive compound that easy to be denatured and less stability. Hence of its lack of characteristic, the character of enzyme is needed to be improved.

The material was prepared by coating magnetite with double silica layer, so that the agglomeration conditions can be avoided. The enzyme support used had different effectiveness from commercial magnetite material. The silica particles that had been coated by TEOS in two stages, then simultaneously used for immobilization of cholesterol oxidase. Figure 1a showed the spectrum of pure commercial magnetite material in the form of Fe–O which was appeared at the peak with a range of 500–700 cm^{-1}. This band was shifted to high wave number compared with the Fe–O–Si bond peak of bulk magnetite at 606.09 cm^{-1}. Figure 1b and c showed the spectrum of synthesized magnetite by single silica layer (Fig. 1b) and magnetite double silica layer (Fig. 1c) which it was indicated the silicon-oxide form in the range between 1200 and 1900 cm^{-1}.

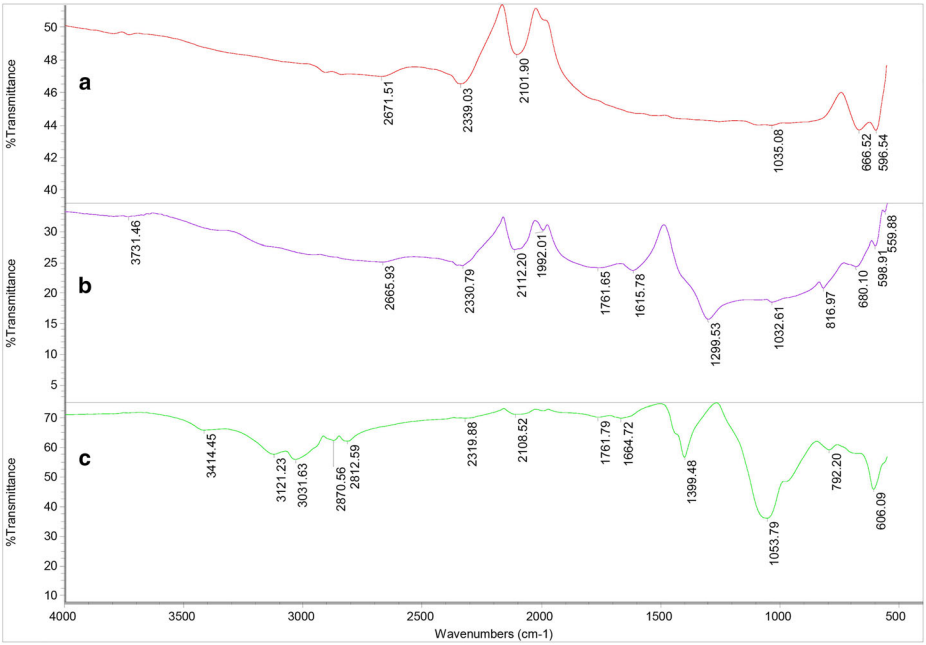

Fig. 1 FTIR spectrum of **a** Fe_3O_4 commercial; **b** Fe_3O_4 single silica layer; and **c**. Fe_3O_4 double silica layer

In this work, a double silica layer has been done by an addition of TEOS after the formation of magnetite template. The double silica layer in formation of Si–O signal was detected at 1053.79 cm^{-1} with broad and sharp peak (Fig. 1c). As shown in Table 1 and Fig. 1, the emerged peaks were identified as Si–O groups, which were appeared in the wavelength at 1299.53 cm^{-1}, 1399.48 cm^{-1}, 1661.78 cm^{-1}, and 1664.72 cm^{-1}. The synthesis process was successfully done as well as identified by the FTIR spectrum.

However, similar results were also expressed in the previous study with the same metal in a form of magnetite particles [15–19]. The double coating of silica layer affected to the peak shape due to the interaction of silicon–oxygen–silicon bonding (Fig. 1c). This interaction performed a strong covalent bond to coat and protect the surface of template. The coated surface with silicon layer will generate hydrophobic surface [17].

Table 1 Functional group of magnetite silicon dioxide

Functional group	Wavelength (cm^{-1})	Wavelength (cm^{-1}) commercial Fe_3O_4	Wavelength (cm^{-1}) Fe_3O_4 double silica layer
Fe–O	500–700	596.54	559.88
Fe–O		666.52	598.91
			680.1
Si–O	1100–1300	–	1299.53
	1500–1800	–	1615.7
			1761.6
O–H	3000–3500	–	3731.46

The morphology of synthesized materials was also investigated (Fig. 2a, b). Based on the SEM results, the magnetite silicon dioxide materials spread uniformly with round and bulky forms (Fig. 2a). However, as seen in bigger magnification, it was shown that the particles stitched each other in a circle shape due to the covalent bonding with the coater materials (Fig. 2b). Silicon dioxide acted as a coater on the surface of magnetite to build a layer. Silicon dioxide has hydrophilic properties so that it allowed the magnetite to stick each other to form uniform small circle shape. The synthesize magnetite particles for enzyme support was also performed in previous study and showed similar results [15, 19].

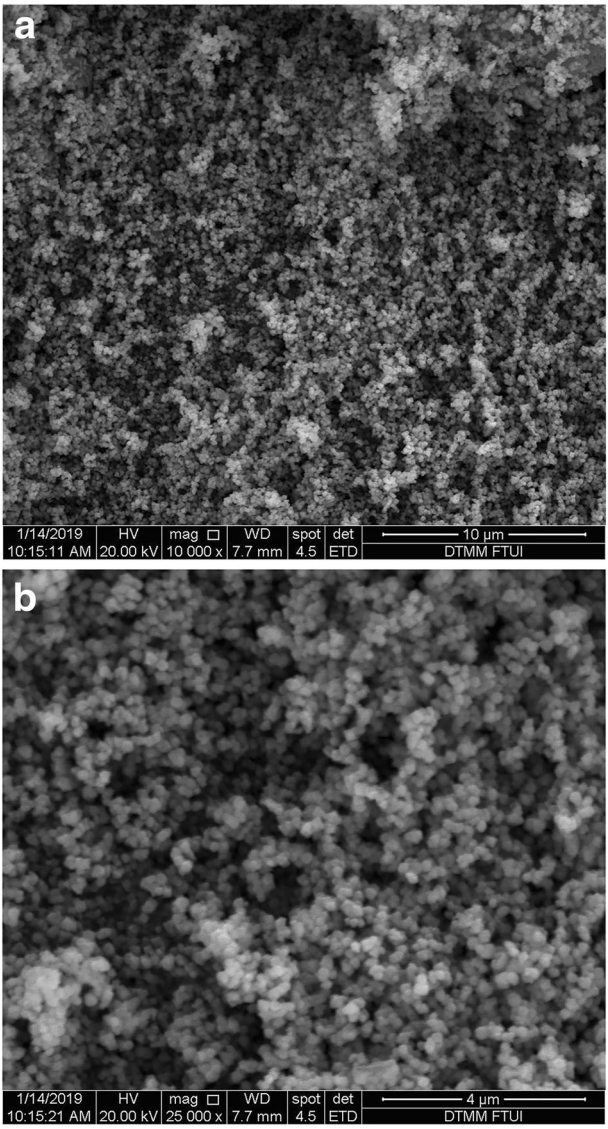

Fig. 2 SEM image of modified magnetite silicon dioxide in different magnification. **a** Magnification × 10.000; **b** magnification × 25.000

Immobilization of Cholesterol Oxidase on Magnetite Silicon Dioxide

An improvement to increase the performance of enzyme has been applied to this study; the cholesterol oxidase was immobilized simultaneously with magnetite silicon dioxide particles. The color of enzyme solution after immobilization changed became cloudy. The protein content loaded on the samples and reached 80.03% of enzyme rate loading. The immobilization technique with crosslinking method was produced final product with enzyme loading activity reaching 55.5 U/g support. Meanwhile, the free enzyme was showed 49.81% of enzyme rate loading. The presence of magnetite silicon dioxide was estimated able to increase the enzyme loading which was activated by glutaraldehyde. The formation of bulky multiple-layered cholesterol oxidase on the surface of magnetite silicon dioxide exhibited the covalent interaction via intermolecular crosslinking. As compared with the free enzyme, there was no coater on the enzyme surface which may cause the enzyme easy to be denatured and showed low enzyme loading. The performance of support for enzyme loading improvement has been published. It showed that enzyme immobilized with bare maghemite (γ-Fe_2O_3) had better activity as compared with free enzyme. In this work, the support used was a combination between magnetite (Fe_3O_4) and silicon dioxide. The difference of support material will also affect the enzyme activity [5, 19, 20].

Glutaraldehyde was used as a crosslinking reagent and produced a strong bonding space between enzyme and support. The amine functional groups of enzymes interact covalently with the oxide groups of silicon dioxide support to form new characteristics. The enzyme was distributed and flowed through the pores of support. Immobilized enzymes are used for testing the oxidation of cholesterol with different interval times. The previous studies revealed that immobilized cholesterol oxidase which is isolated from *Pseudomonas aeruginosa* in silane-modified iron oxide are proven to be able to change the final component of cholesterol and 7-ketocholesterol products to be 4-cholest-3-one and 4-cholesten-3,7-dione. Cholesterol oxidase can change the final composition of oxidized cholesterol. Therefore, the results of cholesterol oxidation products that are formed can decrease along with reduced enzyme activity when reacting [15].

Cholesterol Oxidation by Cholesterol Oxidase

The enzyme concentration factor for cholesterol concentrations were carried out by comparing the enzyme concentration used with different enzyme forms to the incubation time. All oxidation reactions were carried out with aspects of several factors and used the same temperature parameters and rotational speeds. The enzyme concentrations used were 5, 10, and 20 mg/mL with a substrate concentration of 1.94, 3.23, and 6.46 mM. The reaction was controlled by different incubation times, namely 5, 30, 60, 120, and 180 min. Samples were made from each vial incubation time that was made one sample with double injection during analysis using an HPLC instrument.

In Fig. 3, it can be seen that the greater concentration of the enzyme reacted, the greater concentration of the oxidized substrate will obtained (Fig. 3a). At an enzyme concentration of 20 mg/mL, it was effectively oxidizing cholesterol and reached 10% remaining. The effect of enzyme concentration in oxidation reactions has a significant factor in the process of changing enzymatic reactions. In Fig. 3c, free enzyme of 20 mg/mL was able to oxidize 6.46 mM until 40%; somehow the result was similar with the trend of 10 mg/mL of enzyme. It was indicated

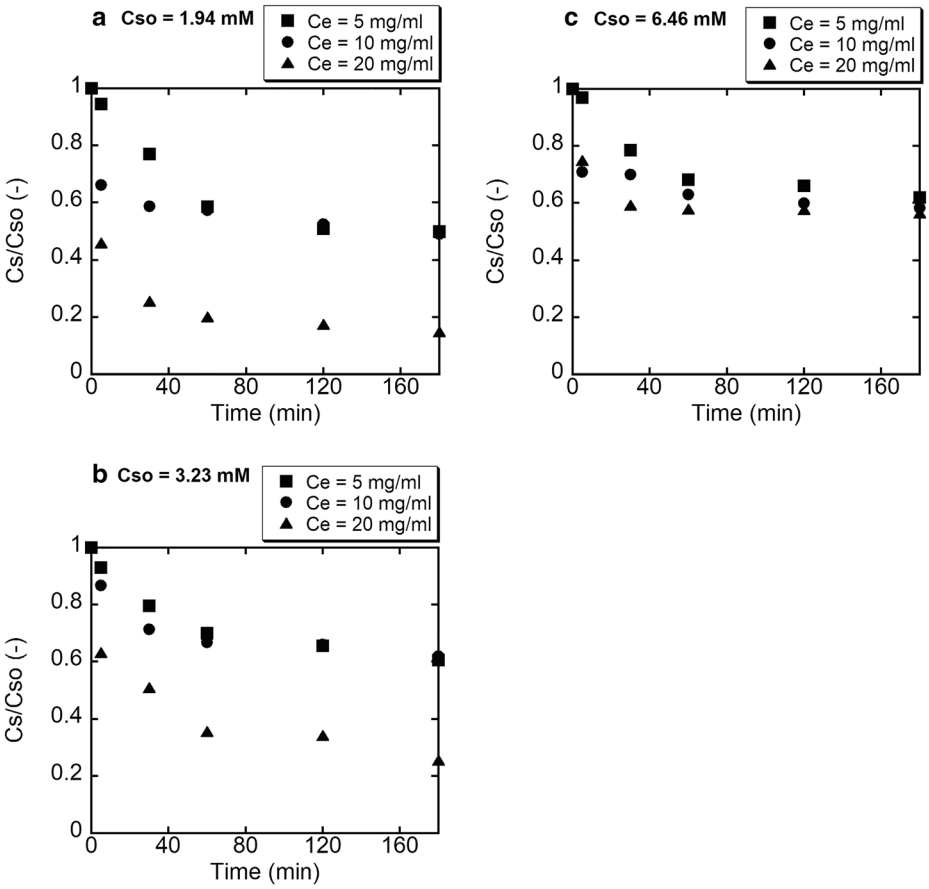

Fig. 3 Effect of enzyme concentration against cholesterol oxidation by free enzyme with initial substrate concentration at **a** 1.94 mM, **b** 3.23 mM and **c** 6.46 mM

that the enzyme had reached the maximum activity in order to oxidize the substrate. Cholesterol which is catalyzed by the enzyme oxidase cholesterol will turn into a cholesterol derivative compound, 4-cholest-3-one [21].

To compare the behavior of free enzyme and immobilized enzyme, an oxidation of cholesterol with immobilized enzyme was also studied. The immobilized enzyme had a good thermal stability at 37 °C. The reaction was involved in warm temperature to protect the activity of enzyme. Immobilized enzyme showed a very significant reduction in cholesterol concentration profile. As shown in Fig. 4, a curve with a negative slope indicating a reduction in cholesterol concentration along with the reaction time of an oxidation reaction. However, overlapping points appeared in the experiment. Immobilized enzymes have great effectiveness, due to the enzyme supported with magnetite material with a large surface area and interaction between enzymes and magnetite silicon dioxide material [22].

The magnetite has been functionalized with amino compounds to covalently immobilized the enzyme via crosslinking. This techniques, affecting to the behavior of immobilized enzyme when reacted with substrate. The catalytic activity of immobilized

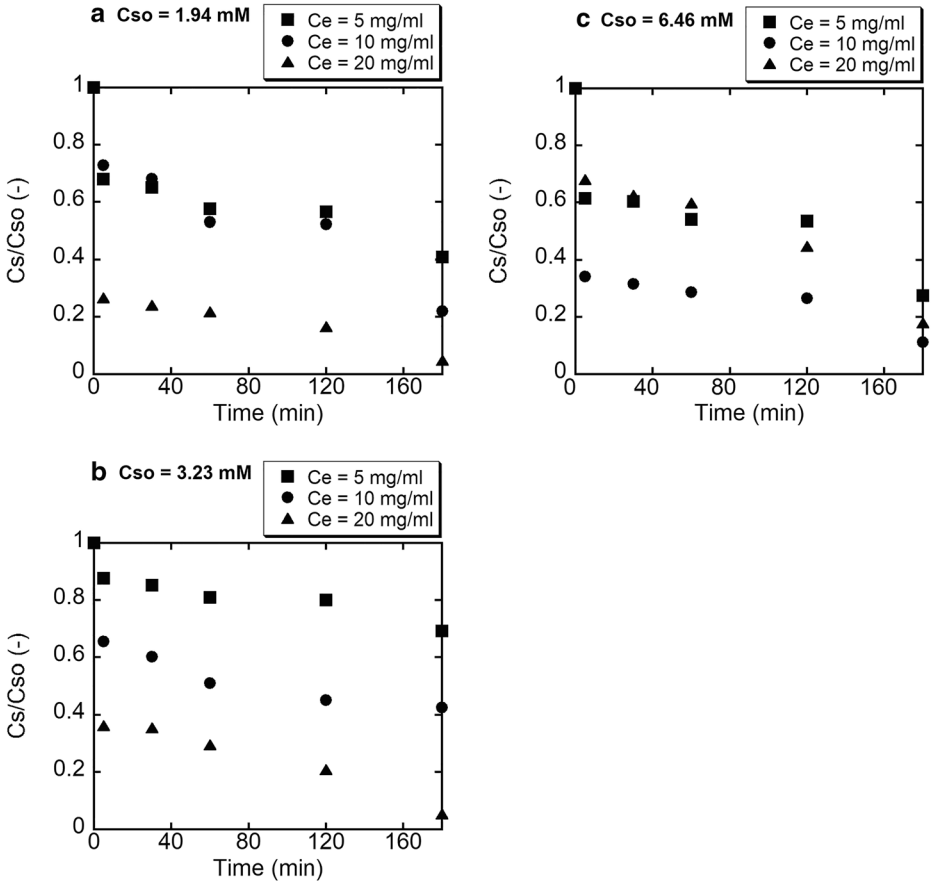

Fig. 4 Effect of enzyme concentration against cholesterol oxidation by cholesterol oxidase immobilized on magnetite silicon dioxide with initial substrate concentration at **a** 1.94 mM, **b** 3.23 mM and **c** 6.46 mM

enzyme was retained and oxidized the substrate simultaneously [23]. During the oxidation reaction, the ability of enzyme to oxidized the substrate depended on the amount of substrate and enzyme. An overlapped happened due to the catalytic characteristic of enzyme which difficult to control. This phenomenon was also called an agglomeration effect of enzyme. The distribution of enzyme was difficult to control and effected to the catalytic performance [24]. Meanwhile, the behavior of free enzyme was much more overlapped since there was no coater in the enzyme. It is supposed that the improvement of enzyme can hardly be explained in the terms of low catalytic activity.

The enzyme form acted as an important effect against the enzyme activity [25]. Based on the oxidation reaction, the immobilized enzyme could interact effectively to the substrate to oxidize the amount of substrate in the reaction mixture. A comparative test between free enzyme and immobilized enzyme was also investigated by equal enzyme concentration (5.12 U/mL in amount of 20 mg/ml) in each experiment. As shown in Fig. 5a, the decreasing of substrate showed that it was greatly oxidized by the enzymes as followed by the reaction time (Fig. 5a). Enzyme support helped the efficiency of enzyme to bind with the cholesterol. In this work, the support was synthesized and

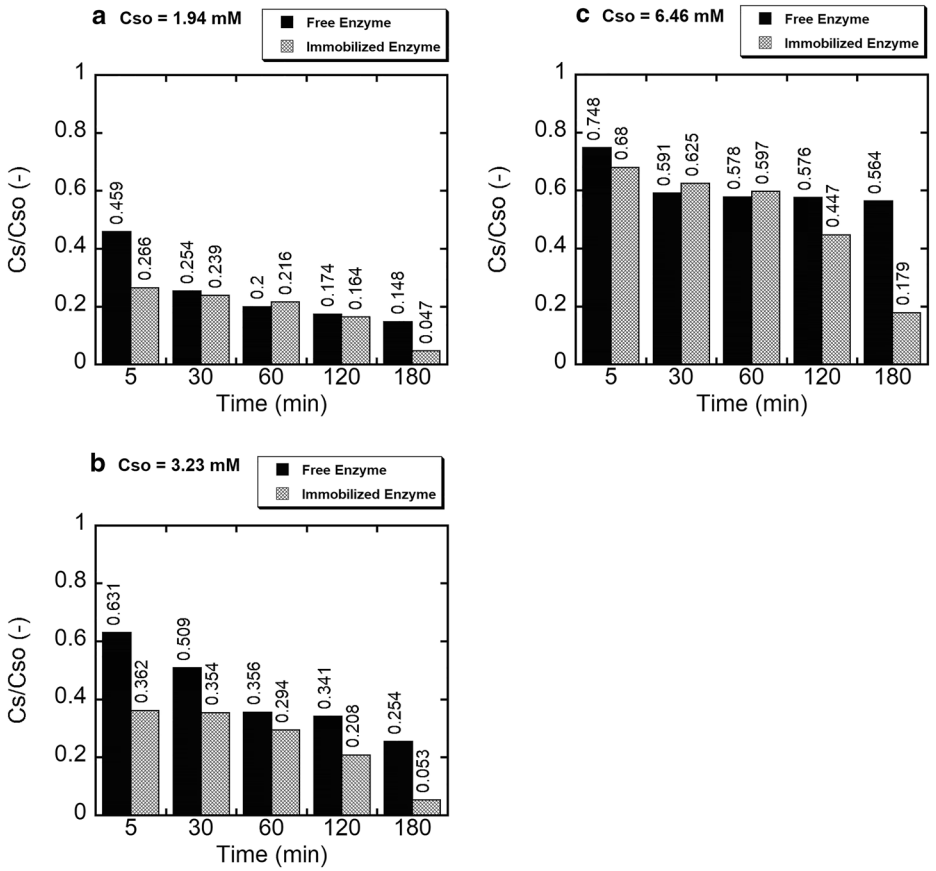

Fig. 5 Effect of enzyme forms at maximum enzyme concentration (20 mg/ml, 5.12 U/mL) against different substrate concentration at **a** 1.94 mM, **b** 3.23 mM, and **c** 6.46 mM

modified with silicon oxide surface. The active surface worked as a gap before the magnetite binding with the cholesterol oxidase. Silicon layer in the particle was interacted each other and formed a bulky particles. This bulky particle helped enzyme to get through the particle and produced specific performance for immobilized enzyme. Based on Huang (2015), the immobilized enzyme had a 90% relative activity for 30 days [5]. The activity of enzyme was preserved and showed good storage stability. An improvement of enzyme with magnetite support is leading to get an advance method for further application.

Conclusions

Cholesterol oxidase has a great potential application to oxidize cholesterol as a substrate. Magnetite silicon dioxide can support the cholesterol oxidase as application for oxidation reaction. Moreover, the concentration of enzyme and substrate will affect to the behavior of oxidation reaction. The larger amount of cholesterol oxidase can oxidize effectively the substrate greater than 60%.

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Compliance with Ethical Standards This chapter does not contain any studies with human participants or animals performed by any of the authors. For this type of study formal consent is not required.

Conflict of Interest The authors declare that they have no conflict of interest.

Nomenclature *Ce*, Concentration of enzyme (mg/mL); *Cs*, Final concentration of substrate (mM); *Cso*, Initial concentration of substrate (mM)

References

1. Neuvonen, M., et al. (2014). Enzymatic oxidation of cholesterol: properties and functional effects of cholestenone in cell membranes. *PLoS One, 9*(8), e103743.
2. Nawaz, M. A. H., et al. (2018). Development of a disposable electrochemical sensor for detection of cholesterol using differential pulse voltammetry. *Journal of Pharmaceutical and Biomedical Analysis, 159*, 398–405.
3. Gao, J., et al. (2019). Simultaneous detection of glucose, uric acid and cholesterol using flexible microneedle electrode array-based biosensor and multi-channel portable electrochemical analyzer. *Sensors and Actuators B: Chemical, 287*, 102–110.
4. Wang, K., et al. (2018). Ratiometric fluorescence sensor based on cholesterol oxidase-functionalized mesoporous silica nanoparticle@ ZIF-8 core-shell nanocomposites for detection of cholesterol. *Talanta, 188*, 708–713.
5. Huang, J., et al. (2015). Immobilization of cholesterol oxidase on magnetic fluorescent core-shell-structured nanoparticles. *Materials Science and Engineering: C, 57*, 31–37.
6. Pashangeh, K., et al. (2017). Biochemical characterization and stability assessment of Rhizopus oryzae lipase covalently immobilized on amino-functionalized magnetic nanoparticles. *International Journal of Biological Macromolecules, 105*, 300–307.
7. MacLachlan, J., et al. (2000). Cholesterol oxidase: sources, physical properties and analytical applications. *The Journal of Steroid Biochemistry and Molecular Biology, 72*(5), 169–195.
8. Vrielink, A., & Ghisla, S. (2009). Cholesterol oxidase: biochemistry and structural features. *The FEBS Journal, 276*(23), 6826–6843.
9. Doukyu, N., & Nihei, S. (2015). Cholesterol oxidase with high catalytic activity from *Pseudomonas aeruginosa*: screening, molecular genetic analysis, expression and characterization. *Journal of Bioscience and Bioengineering, 120*(1), 24–30.
10. Chen, Y., et al. (2013). Immobilization and stabilization of cholesterol oxidase on modified sepharose particles. *International Journal of Biological Macromolecules, 56*, 6–13.
11. Gholivand, M. B., & Khodadadian, M. (2014). Amperometric cholesterol biosensor based on the direct electrochemistry of cholesterol oxidase and catalase on a graphene/ionic liquid-modified glassy carbon electrode. *Biosensors and Bioelectronics, 53*, 472–478.
12. Wisitsoraat, A., et al. (2009). High sensitivity electrochemical cholesterol sensor utilizing a vertically aligned carbon nanotube electrode with electropolymerized enzyme immobilization. *Sensors, 9*(11), 8658–8668.
13. Ghosh, S., Ahmad, R., & Khare, S. (2019). Refolding of thermally denatured cholesterol oxidases by magnetic nanoparticles. *International Journal of Biological Macromolecules, 138*, 958–965.
14. Feng, B., & Liu, Y.-N. (2015). A disposable cholesterol enzyme biosensor based on ferrocene-capped gold nanoparticle modified screen-printed carbon electrode. *International Journal of Electrochemical Science, 10*, 4770–4778.
15. Ghosh, S., et al. (2018). Cholesterol-oxidase-magnetic nanobioconjugates for the production of 4-cholesten-3-one and 4-cholesten-3, 7-dione. *Bioresource Technology, 254*, 91–96.
16. Kulkarni, S. A., Sawadh, P., & Palei, P. K. (2014). Synthesis and characterization of superparamagnetic Fe 3 O 4@ SiO 2 nanoparticles. *Journal of the Korean Chemical Society, 58*(1), 100–104.
17. Zhai, Y., et al. (2016). Magnetic, fluorescence and transition metal ion response properties of 2, 6-diaminopyridine modified silica-coated fe3o4 nanoparticles. *Molecules, 21*(8), 1066.

18. Yang, L., et al. (2018). Modification and characterization of Fe3O4 nanoparticles for use in adsorption of alkaloids. *Molecules, 23*(3), 562.
19. Šulek, F., et al. (2010). Surface functionalization of silica-coated magnetic nanoparticles for covalent attachment of cholesterol oxidase. *Journal of Magnetism and Magnetic Materials, 322*(2), 179–185.
20. Dervisevic, M., et al. (2016). Amperometric cholesterol biosensor based on reconstituted cholesterol oxidase on boronic acid functional conducting polymers. *Journal of Electroanalytical Chemistry, 776*, 18–24.
21. Oliveira, C. L., et al. (2014). Effect of oxidation on the structure of human low-and high-density lipoproteins. *Biophysical Journal, 106*(12), 2595–2605.
22. Pinheiro, P., et al. (2013). Fluorescent magnetic bioprobes by surface modification of magnetite nanoparticles. *Materials, 6*(8), 3213–3225.
23. Li, D., et al. (2019). Screening and analysis of cyclooxygenase-2 inhibitors from the complex matrix: a case study to illustrate the important effect of immobilized enzyme activity in magnetic ligand fishing. *Journal of Pharmaceutical and Biomedical Analysis, 175*, 112,795.
24. Kim, M. G., & Lee, S. B. (1996). Enhanced catalytic efficiency of enzymes in organic media with the addition of the solid support: effect of silica gel on reaction rates and enzyme agglomeration. *Journal of Molecular Catalysis B: Enzymatic, 2*(2–3), 127–140.
25. Temani, M., Baccar, Z. M., & Mansour, H. B. (2014). Activity of cholesterol oxidase immobilized on layered double hydroxide nanomaterials for biosensor application: *Acacia salicina* scavenging power of hypercholesterolemia therapy. *Microelectronic Engineering, 126*, 165–168.

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