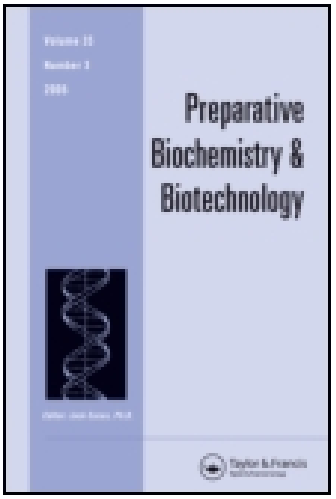

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Mustafa Teke [a]
[a] Chemistry Department, Science Faculty , Muğla Sıtkı Koçman University , Muğla , Turkey
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DEVELOPMENT OF A NEW BIOSENSOR FOR DETERMINATION OF CATALASE ACTIVITY

Mustafa Teke

Chemistry Department, Science Faculty, Muğla Sıtkı Koçman University, Muğla, Turkey

□ *Catalase is one of the major antioxidant enzymes that catalyzes the hydrolysis of H_2O_2. The aim of this study was to suggest a new method for the assay of catalase activity. For this purpose, an amperometric biosensor based on glucose oxidase for determination of catalase activity was developed. Immobilization of glucose oxidase was made by a cross-linking method with glutaraldehyde on a Clark-type electrode (dissolved oxygen probe). Optimization and characterization properties of the biosensor were studied and determination of catalase activity in defined conditions was investigated in artificial serum solution. The results were compared with a reference method.*

Keywords catalase, Clark electrode, glucose oxidase biosensor

INTRODUCTION

Catalase (EC 1.11.1.6) is one of the major antioxidant enzymes that protects the cell against the oxidative damage at high concentrations of H_2O_2.[1] The catalysis mechanism of catalase is as follows:

$$H_2O_2 + H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

The enzyme consists of four identical subunits. Each of the monomers contains a heme prosthetic group.[2,3]

In 1948 and 1952, Takahara et al. reported that Japanese acatalesemia (also known as Takahara disease) patients suffered from gradual oral gangrene owing to catalase deficiency in blood.[4,5] In 1966, Ogata et al. suggested lack of catalase in blood to be the cause of the disease.[6] A high incidence of diabetes mellitus was reported by Goth et al. in Hungarian acatalasemia patients[7,8] and was associated with catalase enzyme mutations.[9]

Address correspondence to Mustafa Teke, Muğla Sıtkı Koçman University, Science Faculty, Chemistry Department, 48000, Muğla, Turkey. E-mail: mteke@mu.edu.tr; mustafateke42@hotmail.com

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Recently, Goth et al. and Masuoka et al. reported that the diabetes mellitus was associated with low catalase activity in blood.[10,11] Besides this, high catalase activity was searched out in patients with hyperthyroidism due to Graves' disease.[12–14] Furthermore, catalase activity in plasma of patients is assigned to be one of antioxidant markers.[15] Therefore, determination of catalase activity in erythrocyte, plasma, and serum is very important in the early diagnosis of diseases.

There are a few methods that focus on spectrophotometric methods for determination of catalase activity.[16–19] This is one of the classical methods because the catalase is a hydrolase enzyme. Only in one literature report was catalase activity determined using a three-electrode photoelectrochemical cell.[20] Although these methods successfully have demonstrated catalase activity determination, most of them involve expensive instruments and may not be suitable for routine analysis.

For this article, a Clark-type oxygen electrode based on immobilized glucose oxidase was utilized to assay the catalase activity. In the reaction cell and the electrode surface, reactions are as follows:

Step 1:

$$\beta\text{-D-Glucose } (1\,\text{m}M) + O_2 \xrightarrow{\text{Glucose oxidase}} \text{D-glucono-1, 5-lactone} + H_2O_2$$

to remove dissolved oxygen in the reaction medium.

Step 2:

$$2H_2O_2\ (10\,\text{m}M) \xrightarrow{\text{Catalase}} 2H_2O + O_2{}^*$$

Step 3:

$$\beta\text{-D-Glucose}(10\,\text{m}M) + O_2{}^* \xrightarrow{\text{Glucose oxidase}} \text{D-glucono-1, 5-lactone} + H_2O_2$$

The asterisk in the equation in steps 2 and 3 shows the oxygen used for the determination of catalase activity during the enzymatic reactions.

Measurements were achieved by standard curves. Using the biosensor, it became possible to analyze catalase activity at the levels of nanograms. β-Galactosidase activity assay was carried out successfully with a method based on a similar principle, reported by Sezgintürk et al.[21]

EXPERIMENTAL

Chemicals

Sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), and sodium citrate were purchased from E. Merck (Germany). Glucose oxidase (Type-II-S-*Aspergillus niger*; 26.82 U/mg), gelatin (type 2, 225 Bloom), catalase (bovine liver, 3390 U/mg), and glutaraldehyde (Grade II, 25%) were purchased from Sigma (St. Louis, MO). All chemicals were used at analytical grades.

Apparatus

YSI 58 model oxygen meters (wide measurement range: 0 to 20 mg/L, exceptional accuracy of ±0.03 mg/L, temperature display) and YSI 5718 series dissolved oxygen (DO) probes (YSI Co., Inc., Yellow Springs, OH) were used. A water bath was used for all measurements at constant temperature in the reaction cell by circulating water and preparation of bioactive material (Nüve, BM402, Turkey). A pH meter with electrode (WTW 330İ/SET, Germany) and a magnetic stirrer (VELF Scientifica, 171280, Germany) for preparing buffer solutions were used.

Preparation of the Bioactive Layer Material

Initially, a dissolved oxygen probe was covered to construct the biosensor with oxygen sensitive Teflon membrane by O-ring. Then the Teflon membrane was pretreated with 0.5% sodium dodecyl sulfate (SDS) in pH 7.5, 50 m*M* phosphate buffer, to minimize the tension on the Teflon membrane. After this step, 26.4 U glucose oxidase and 5 mg gelatin were dissolved in 200 μL of 50 m*M* phosphate buffer, pH 7.0, at 38°C for 10 min. The enzyme and gelatin mixture was dispersed over the probe and allowed to dry at 4°C for 30 min. The glucose oxidase-coated probe was submerged in 5% (v/v) glutaraldehyde prepared in pH 7.5, 50 m*M* phosphate buffer, for 5 min. After the biosensor was washed gently with distilled water, it was stored without coming into contact with some distilled water in a flask at 4°C.

Measurement Procedure

First, the biosensor was immersed into the reaction cell containing working buffer (pH 7.5, 50 m*M* Na phosphate buffer), which was fixed with the magnetic stirrer. After 5 min dissolved oxygen concentration was equilibrated between the biosensor and working buffer. Afterward, all measurements were performed in three steps. At step 1, 1 m*M* glucose was

injected to remove dissolved oxygen into the reaction cell and after removal of oxygen; at step 2, catalase was injected into the reaction cell contained 10 m*M* H_2O_2. The dissolved oxygen concentration started to increase and 10 min later, owing to the enzymatic reaction, it reached a constant dissolved oxygen concentration. Increased dissolved oxygen concentration was recorded. At step 3, 10 m*M* glucose was added into the reaction cell. The dissolved oxygen concentration started to decrease due to enzymatic reaction of glucose oxidase, and after 5 min it reached a constant dissolved oxygen concentration. Decreased dissolved oxygen concentration was recorded. All values were performed by monitoring the change of dissolved oxygen concentration related between catalase (step 2) and glucose oxidase (step 3) activities.

RESULTS AND DISCUSSION

Optimization of the Biosensor Component

In these studies, glucose oxidase activity, gelatin amount on the probe (oxygen electrode) surface, and glutaraldehyde percentage on the biosensor were investigated. All values were achieved by drawing each of the standard graphics (the amount of dissolved oxygen depending on catalase activity between 5 ng/mL [1.7×10^{-2} U/mL] and 60 ng/mL [20.4×10^{-2} U/mL] obtained under the conditions of glucose oxidase activity [5.4 U, 13.4 U, 26.8 U, 40.2 U], gelatin amount [2.5 mg, 5 mg, 7.5 mg] and glutaraldehyde percentage [2.5, 5, 7.5%]). The biosensor prepared with 26.8 U glucose oxidase had the widest linear range for catalase activity. For the lower and higher amounts than 5 mg gelatin (2.5 and 7.5) the biosensor responses were not impaired remarkably. Besides this, at the concentrations higher than 5% of glutaraldehyde, the biosensor response decreased. Because the active sites of glucose oxidase were blocked by cross-linking between the enzyme and gelatin. At the concentrations lower than 5% of glutaraldehyde, the detection limit of the biosensor increased. Because a sufficient amount of the enzyme on the electrode surface was not bound, the optimization studies indicated that the best responses were obtained when the quantities of 26.8 U glucose oxidase activity, 5 mg gelatin, and 5% glutaraldehyde percentage were used.

Optimum Temperature

In this biosensor system for catalase activity assay, there were two different enzymes in the measurement process. Thus, determination of optimum temperature for the biosensor was primary. One of them is catalase as

sample, and the other is glucose oxidase from the biosensor. Consequently, measurements were designated between 20 and 45°C. The biosensor responses increased with changed temperature. As shown in Figure 1, the highest value was observed at 35°C. At the temperatures above this, the biosensor responses decreased with increasing temperature.

Optimum pH

Determination of the pH dependency of the biosensor was also one of the most important biosensor parameters. Optimum pH values for free glucose oxidase and free catalase are between 4 and 7[22] and between 6 and 8,[23] respectively. For this purpose, 50 m*M* citrate (pH 4.0–5.5) and sodium phosphate (pH 6.0–8.5) buffers were used in experiments.

According to the results, the best biosensor response was obtained at pH 7.5, sodium phosphate buffer. As can be seen from Figure 2, the biosensor signal was also high at pH 5.5, sodium citrate buffer. This result probably shows that glucose oxidase is more efficient for the biosensor response.

Effect of Buffer Concentration

The effect of buffer concentration was also studied. The biosensor response was affected slightly by buffer concentration. For this aim, pH 7.5 sodium phosphate (10–25–50–75–100 m*M*) buffers were used in the experiments. The best biosensor signal was obtained at 50 m*M* phosphate buffer. The biosensor signals recorded decrease at below and above this concentration due to the changes in the enzyme structure.

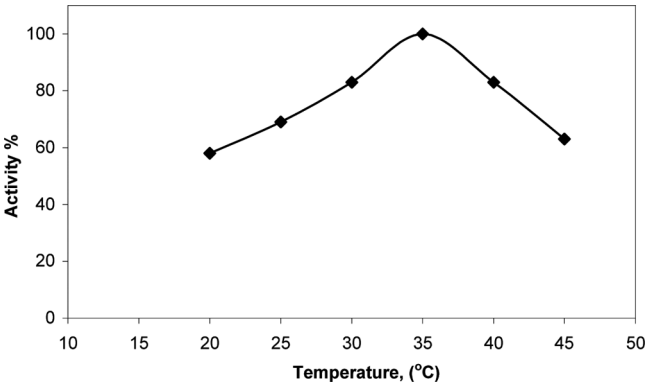

FIGURE 1 Temperature effect on the biosensor response. Working conditions: amounts of glucose oxidase, catalase, gelatin, and percentage of glutaraldehyde were kept constant as 26.8 U, 20.4×10^{-2} U, 5 mg, and 5%, respectively. Working buffer was 0.05 m*M* sodium-phosphate solution, pH 6.0, and contained 10 m*M* H_2O_2 substrate of catalase.

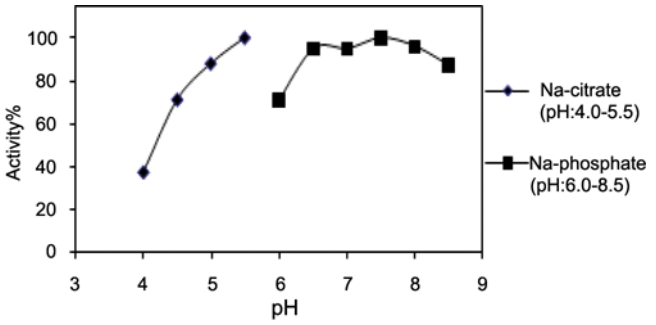

FIGURE 2 pH effect on the biosensor response. Working conditions: amounts of glucose oxidase, catalase, gelatin, and percentage of glutaraldehyde were kept constant as 26.8 U, 20.4×10^{-2} U, 5 mg, and 5%, respectively. All buffers was 0.05 m*M*.

CHARACTERIZATION STUDIES OF THE BIOSENSOR

Calibration Graph for Catalase Activity

A calibration curve for catalase activity is presented in Figure 3. The linear concentration range was between 1.7×10^{-2} and 20.4×10^{-2} U/mL with $y = 23.84x + 0.5347$ and $R^2 = 0.9817$. In the studies, a commercial bovine liver catalase was used. Its activity amount was at the level of 3390 U/mg. The detection limit for the biosensor was 5 ng/mL (1.7×10^{-2} U/mL) of catalase.

Repeatability

For the repeatability measurements, 10 replicate determinations of 20.4×10^{-2} U/mL of catalase standard solution were obtained. The average

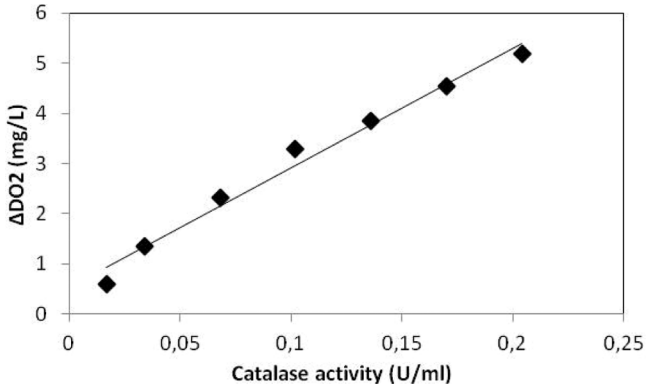

FIGURE 3 Catalase activity calibration graph. Biosensor components of glucose oxidase activity, gelatin amount, and percentage of glutaraldehyde were kept constant as 26.8 U, 5 mg, and 5%, respectively. Working buffer was 0.05 *M* Na phosphate solution, pH 7.5, and contained 10 m*M* H_2O_2 substrate of catalase, $T = 35°C$.

TABLE 1 Catalase Activity Determination (U/mL) in Artificial Serum Solution by the Biosensor and by a Spectrophotometric Reference Method[17]

Added	Found by the Biosensor	Found by the Reference Method	Recovery (%)	Relative Difference (%)
0.204	0.210	0.195	97	7

value (X), standard deviation (SD), and variation coefficient (CV) were as follow: 20.45×10^{-2} U/mL, $\pm 8.4 \times 10^{-4}$ U/mL and 0.41%, respectively. As a result, the biosensor had analytical features in terms of repeatability.

Thermal Stabilities

For this purpose, catalase was incubated at different temperatures, 25, 30, 35, 40, and 45°C, for 5 hr. There was no activity change in the enzyme incubated at 25°C. However, the enzyme incubated at the temperatures of 30, 35, and 40°C lost 5%, 8%, and 18% of its initial activity, respectively. In addition, the biosensor lost about 67% of its initial activity after an incubation period of 5 hr at 45°C due to disruption of the bioactive layer.

Operational Stability

This property of the biosensor was determined from consecutive values of the responses to catalase activity standard, 20.4×10^{-2} U/mL. These measurements were obtained at 35°C. After 25 measurements, 75% of initial activity had been retained. On the other hand, there was no activity change in the enzyme at the end of the 12th measurement. As a result, the biosensor was quite good in terms of operational stability.

SAMPLE ANALYSIS

For this purpose the catalase activity of artificial serum solution was detected by using the biosensor. It contained 2.5 mM urea, 0.1% human serum albumin, 4.5 mM KCl, 5 mM $CaCl_2$, 1.6 mM $MgCl_2$, and 145 mM NaCl and 4.7 mM D(+)-glucose. The results were compared with a reference method.[17] Consequently, as shown in Table 1, the proposed biosensor is fairly effective for detection of catalase activity.

CONCLUSION

In this study, an enzyme biosensor was demonstrated to investigate the activity of catalase. For this purpose, the biosensor was optimized and

characterized. Especially, it can be used for monitoring catalase. Moreover, the biosensor has also important advantages in terms of low detection limit (1.7×10^{-2} U/mL or differently 5 ng/mL), linear range, operational stability, and simple and cheap measurement method.

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