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Biosensor application of extracellular catalase from *Ganoderma lucidum* AVK-1 and *Acinetobacter calcoaceticus* AV-6 and Comparative study of commercial catalase from Bovine liver catalase

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Abstract: *An amperometric biosensor based on catalase enzyme was developed for the investigation of the effect of calcium ions on the activity of the enzyme. Calcium plays an activator role for the catalase enzyme that catalysis the degradation of hydrogen peroxide to O_2 and H_2O. Determination method of the effect of calcium ion on the activity of the enzyme was based on the assay of the differences on the responses of the biosensor in the absence and the presence of calcium in the reaction medium. The biosensor had a linear relation to calcium concentrations and good measurement correlation between 0.05 and 15 mM with 3 min response time. PPB–HCl buffer (pH 6.8; 20 mM) and 37ºC were obtained as the optimum working conditions. In the application studies, the biosensor was used determination of calcium level of real samples such as cow milk, Aavin (green) and MD water. In the characterization studies of the biosensor some parameters such as reproducibility, substrate specificity, operational and storage stability were carried out. Finally, by using the biosensor developed and enzymatic-spectrophotometric method alcohol concentrations of some alcoholic drinks were determined and results were compared.*
Keywords: *Acinetobacter calcoaceticus, Ganoderma lucidum, Catalase, Biosensor, Milk and calcium*

I. Introduction

Calcium (Ca) is the most abundant mineral in the human body. It is important for intracellular metabolism, bone growth, blood clotting, nerve conduction, muscle contraction and cardiac functions [1 & 2]. Calcium and calcium salts are also very important minerals used in the food industry. Different calcium salts have been studied for decay prevention, sanitation and nutritional enrichment of fresh fruits and vegetables. Calcium carbonate and calcium citrate are the main calcium salts added to foods in order to enhance the nutritional value. Calcium lactate, calcium propionate and calcium gluconate have shown some of the benefits of the use of calcium chloride, such as product firmness improvement, and avoid some of the disadvantages[3]. Also, the use of calcium salts other than calcium chloride could avoid the formation of carcinogenic compounds (chloramines and trihalomethanes) linked to the use of chlorine [4].

II. Experimental

Preparation of catalase enzyme

Ganoderma lucidum AV-1 and *Acinetobacter calcoaceticus* AV-6 those organisms were used for this study. Synthesis and extraction of catalase was purified and applied for biosensor technology and commercial Bovine Liver Catalase also tested.

Chemicals

Catalase (hydrogen peroxide: hydrogen peroxide Oxidoreductase). (EC 1.11.1.6.) from bovine liver 1.9 U mg^{-1}, $CaCl_2$, $NaCl_2$, KCl_2, $MgSO_4$, $NiCl_2$, $CuSO_4$, $MnCl_4$, KH_2PO_4, K_2HPO_4, calf skin gelatin, glutaraldehyde (25%), Na EDTA, and all other chemicals were purchased from Sigma Chemical Co. (USA). All solutions were prepared with double distilled water just before their use.

Apparatus

In these experiments, a YSI Model 5300 Biological Oxygen Meter (Yellow spring instrument co. inc., Yellow spring Ohima 45387 USA) with 0.01-mg/l dissolved oxygen (DO) concentration sensitivity, YSI 5700 Model DO probes (with YSI 5740 cable) as transducers, standard teflon membranes (YSI, Yellow Springs, OH, USA), Accupipet P100 and P1000 automatic pipets (USA), Yellow-Line magnetic stirrer (Germany) and Nuve model thermostat (TR) were used.

Preparation of the biosensor

First of all, a DO probe was covered with a standard teflon membrane using an O-ring and then the membrane which is sensitive for oxygen was pretreated with 0.5% SDS (sodiumdodecylsulphate) in phosphate buffer (50 mM, pH 7.0) to reduce the tension on the membrane surface of the DO probe. After this step, 250 µl of catalase enzyme solution and gelatin were mixed and dissolved at 38ºC for a few minutes. Two-hundred microlitre of the solution was spread over the DO probe membrane surface and allowed to dry at 4 ºC for 30 min. At the end of the time, the bioactive layer was treated with glutaraldehyde (2.5%, in phosphate buffer; 50 mM, pH 7.0) for 3 min to form chemical covalent bonds (Schiff bases) between gelatin, enzyme and glutaraldehyde molecules for the immobilisation of the enzyme on the surface of the DO probe.

Measurements

In the reaction, catalase converts hydrogen peroxide to hydrogen dioxide and carbon dioxide in the presence of oxygen. There is an intermediate surface between the bioactive layer and the teflon membrane of the DO probe and during

the enzymatic reaction dissolved oxygen concentration in the intermediate surface decreased relative to the substrate concentration added into the reaction medium. The measurements with the developed biosensor were carried out at steady-state conditions. DO is the differences of the dissolved oxygen concentration when the substrate is not in the reaction medium and after addition of substrate into the reaction medium to obtain a new steady-state DO concentration. It is well known that calcium is a cofactor for catalase and it plays an activator role for the catalase so when the calcium was injected into the reaction medium it increased the activity of the enzyme and in this case the dissolved oxygen concentration changed relative to the calcium concentration added into the reaction medium.

The principle of the measurement of the biosensor was based on the determination of these changes in the dissolved oxygen concentration related to calcium concentrations used in the enzymatic reaction. As a result, the differences between the first and the final dissolved oxygen concentrations related to calcium concentrations were detected by the biosensor to obtain a standard curve for the determination of calcium. All the measurements were carried out at 37º C using a thermostatic reaction cell and oxygen saturated tris–HCl buffer (50 mM, pH 7.0).

III. Results and discussion

Detection of calcium effect as an activator on the biosensor responses

At the beginning of the study, some experiments were carried out for the determination of the effect of calcium as an activator on the catalase enzyme biosensor. For this purpose firstly, the developed biosensor was used only for hydrogen peroxide detection using standards with concentration between 1 and 10 mM in the absence of calcium and a linear curve was obtained. After that, by using the same hydrogen peroxide standards but in the presence of 5 mM calcium a new standard curve was obtained. Fig. 1 shows the results obtained from the experiments. According to the figure the biosensor responses increased very efficiently in the presence of calcium.

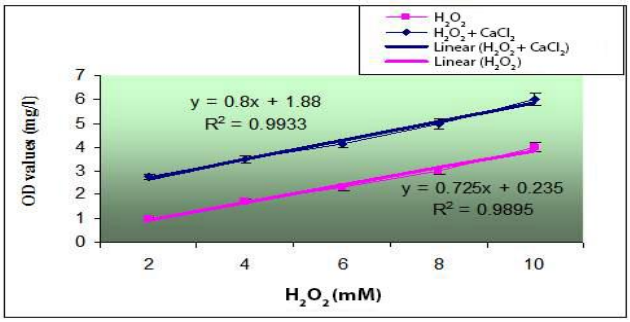


Figure1. Effect of $CaCl_2$ with catalase on break down of H_2O_2

Optimisation of the bioactive surface of the biosensor

Effect of the enzyme activity on the biosensor response Different enzyme amounts were used for determination of the effect of the enzyme activity on the biosensor response. For this purpose, three biosensors which contain 0.5, 0.75 and 1.0 Ucm^{-2} catalase were prepared by immobilising with gelatin (5.31 mg $cm^{2)}$ and glutaraldehyde (2.5%).

Estimation of calcium using enzyme electrode biosensor

Catalase, the most useful calibration curve was obtained. Calcium was detected with a linear range between 1 and 10 mM concentrations by this biosensor. Increase in the catalase activity from 0.75 to 1.0 cm^2 contained 0.75 U cm^{-2} resulted in higher biosensor responses. When the bioactive layer of the biosensor contained 0.5 and 1.0 U cm^{-2} activity of catalase we didn't obtain any suitable standard curve for calcium. In this case, if we consider the results obtained from the experiments it can be said that the most suitable biosensor responses were obtained by the biosensor which contained 0.75 U cm^{-2} activity of catalase.

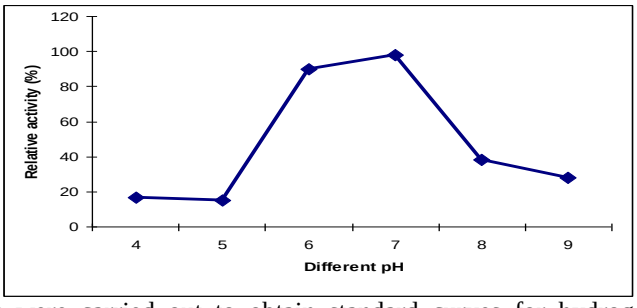
Detection of the effect of gelatin amounts on the biosensor response

To determine the effect of the amount of gelatin on the biosensor response different gelatin amounts were used in the construction of the biosensor. Biosensors contained 0.25, 0.50, 0.75 and 10 mg cm^{-2} gelatin and 0.75 U cm^{-2} catalase. All the biosensors were immobilised by 2.5% of glutaraldehyde. The measurements were made in order to obtain standard curves for calcium. The most suitable curve was obtained with the biosensor prepared using 10.0 mg cm^{-2} gelatin amounts.

From the experiments when the gelatin amount increased from 2.5. to 7.5 mg cm^{-2}, we obtained higher biosensor responses but there was deviation from linearity. Decreases of the gelatin amounts from 0.50 to 0.75 mg cm^{-2} results in a lower biosensor response. The reason of this effect was the reducing in the forming of cross-linked bonds between enzyme–gelatin–glutaraldehyde. Therefore the enzyme escaped from the bioactive layer. From the results it can be uttered that the most suitable biosensor responses were obtained by the biosensor which contained 10.0 mg cm^{-2} gelatin.

Effect of the percentage of glutaraldehyde on the biosensor response

For the determination of the effect of the percentage of glutaraldehyde on the biosensor response different glutaraldehyde amounts were used in the construction of the biosensor. For this purpose we prepared biosensors which contain 0.75 U cm^2 catalase and 0.75 U cm^{-2} gelatin. The biosensors were treated with 1.25%, 2.5% and 3.75% glutaraldehyde solution prepared in phosphate buffer (50 mM, pH 7.0) for the immobilisation.



After the immobilisation procedure, experiments were carried out to obtain standard curves for hydrogen

peroxide using the prepared biosensors.

Experiments showed that the higher biosensor responses were observed at 2.5% glutaraldehyde percentage. The biosensors those were prepared with 1.25% and 3.75% glutaraldehyde, showed lower biosensor responses. As a result of the good linear range and high biosensor responses the biosensors were prepared with 2.5% glutaraldehyde.

Optimisation of working conditions

Effect of pH on the biosensor response

To determine the effect of the pH value on the biosensor response different buffer systems were investigated. For this aim 50 mM con- -centration of citrate (pH 5.0–6.0), tris–HCl (pH 7.0– 8.0) and glycine (pH 9.0–10.0) buffers were used in the experiments.The optimum pH value was determined to be 7.0 Fig. 2. From the experiments below and above this pH value decreases in the biosensor responses were observed. If we consider the optimum pH value (pH 7–8) of the free catalase it can be uttered that the immobilisation procedure did not affect the optimum pH value of the enzyme.

Figure 2.Effect of different pH on the activity of catalase for break down of H_2O_2

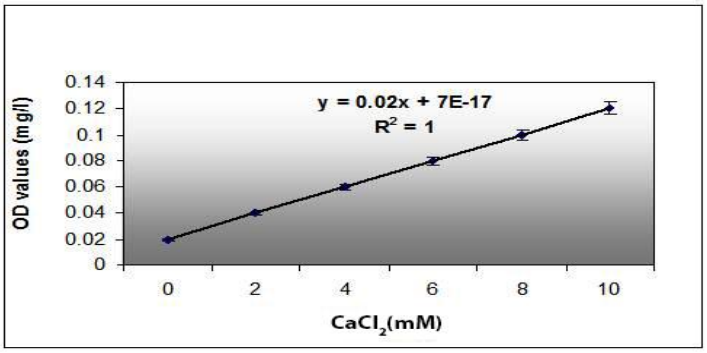


Figure 3. Standard curve for calcium determination

Effect of temperature on the biosensor response

The enzyme activity depends on the temperature and the medium conditions. For determination of the effect of temperature on the biosensor response, experiments were carried out between 15 and 40 ºC. The highest biosensor responses were observed at

37 ºC. Below and above this degree, decreases in the biosensor responses that probably resulted from the changes in the enzyme structure, were recorded.

Analytical characteristics of the biosensor

Linear range of the biosensor

The results obtained for the determination of detection limits for calcium are given in Fig. 3. When we consider the figure, it can be said that the biosensor responses depended linearly on the calcium concentration between 1 and 10 mM with ($y = 0.02x + 7E-17$), and $R^2 = 1$ the detection limit of the biosensor was determined to be 1 mM.

Reproducibility

The reproducibility of the biosensor was also investigated for 5 mM calcium concentration (n=7). The average value (x), the standard deviation (SD) and variation coefficient (CV %) were calculated to be 5.1 mM, ±0.106 mM and 2.07%, respectively.

Effect of some metal ions on the activity of catalase

In order to determine the effect of different compounds on the catalase activity some experiments were carried out using 5 mM calcium and various substances such as $CaCl_2$, $NaCl_2$, KCl_2, $MgSO_4$, $NiCl_2$, $CuSO_4$ and $MnCl_2$ at the same concentration with calcium in the presence of 5 mM hydrogen peroxide. The increase in the biosensor response obtained with calcium was compared to other biosensor responses obtained in the presence of the other substances.

In the other words, for the investigation of these metal ions on the catalase activity in the presence of calcium ion, some experiments were made. Table 1 also shows these results obtained from the experiments.

According to the results, although $MnCl_2$, KCl_2, $MgSO_4$ and $NiCl_2$ played activator role on the catalase activity in the absence of calcium ion, they showed negative effects on the catalase activity in the presence of calcium.

Table 1 Detection of the effects of some metal ions on the activity of catalase in the presence and absence of calcium ion.

Metal ions	Response%
$Cacl_2$	100
$MgSO_4$	80
$NiCl_2$	70
Nacl	30
KCl	10
$CuCl_2$	05

Values are triplicates

Application

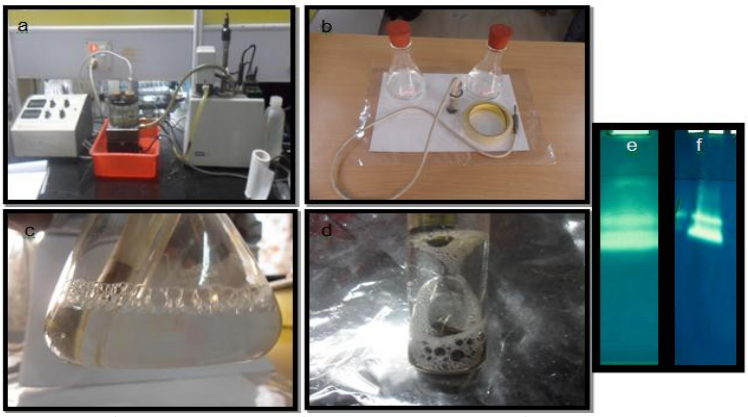
In this section of the study the calcium content of some drinks were detected by using the biosensor. The results obtained from the biosensor were compared to results obtained using a reference procedure [5]. For the same samples in order to complete the validation of the new method. For this goal, some drinks such as milk, water and mineral water which

contains different quantity of calcium, were used. Results obtained from the experiments were given in Table 2. From the experiments when we compare the results of two methods it can be said that calcium in the drinks can be determined sensitively by using the biosensor.

Table 2. Determination of Calcium level of some commercial, raw milk and liquid by using the amperometric biosensor method. (E. Akyilmaz et al., 2009)

Sample	Reported (mg/l)	Found (mg/ml by the biosensor)	Recovery (%	SDS	
Milk-1	120	110	99	±700	
Milk-2	127	126	99	±1.410	
Milk-3	170	167	98	±0.866	
GD- water	335	133	98	±1.000	
Hot water	80	290	101	±0.100	

Values are triplicates



Overal experiment figure Comparison of biosensor application of commercial catalase and purified *Acinetobacter calcoaceticus* AV-6 catalase

a. Biosensor unit of YSI model 5300
b. Oxygen electrode with thermo static cell
c. Enzyme coating on oxygen electrode and treated with SDS (reduce the tension)
d. Thermostatic cell of oxygen electrode during reaction time tested for activity (2µL)
e. *Acinetobacter calcoaceticus* AV-6 catalase on activity staining
f. Bovine liver catalase activity expression on native gel

I. Conclusion

In this study, an amperometric biosensor was developed in order to investigate the effect of calcium on the activity of catalase enzyme. From the experimental studies we detected a positive effect of calcium on the enzyme activity and this effect increased in higher calcium concentrations. By using the biosensor we detected a linear concentration range for calcium in the presence of constant concentration of hydrogen peroxide. The biosensor is really original for Ca determination especially liquid samples and there is no any study like this in the literature. It does not need any expensive equipments, materials or laboratory conditions. The biosensor is portable so it can be used in everywhere for Ca analysis. For all liquid samples it is not necessary any pre-treatment for the samples except dilution (if it is necessary) in the Ca determination. The biosensor developed can be used not only food samples also clinical purpose. In dealing with a large number of samples, the biosensor is rapid, accurate, and precise and with low operation cost is required. By using the biosensor because of the specificity of the enzyme we can determine calcium concentration in the presence of the other metal ions such as Cu+and K_2+, Mg^{2+}, Ni2+, Na2+, Mn2. Reproducibility of the biosensor is very well and from the application studies of real samples it can be said that the biosensor can be used as a sensitive alternative method for analysis of calcium.

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