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Do copper ions activate tyrosinase enzyme? A biosensor model for the solution

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ABSTRACT

Some metal ions play a cofactor role for the activity of tyrosinase enzyme and one of them is copper ion. In this study an amperometric biosensor was developed in order to investigate the effect of the copper ions on the activity of tyrosinase enzyme. In the construction of the biosensor tyrosinase enzyme was immobilized on a Clark-type dissolved oxygen probe which was covered with a oxygen sensitive teflon membrane, by using a chemical covalent immobilization method based on gelatine and bifunctional reagent, glutaraldehyde. The principle of the measurement was based on the determination of the differentiation of dissolved oxygen level in the enzymatic reaction catalyzed by tyrosinase in the absence and the presence of copper ions. Differences between the dissolved oxygen concentrations were related to copper ion concentration which was added in to the reaction medium. The biosensor response depends linearly on copper ion concentration between 2.5–20.0 μM with a response time 1 min. The detection limit of the biosensor is 0.95 μM.

In the optimization studies of the biosensor, the most suitable amounts of tyrosinase, gelatin and glutaraldehyde ratio were determined to be 69.0 U/cm^{-2}, 4.21 mg/cm^{-2}, and 2.5%, respectively. In the optimization studies of the biosensor, phosphate buffer (pH 7.0 ,50 mM) and 30 °C were detected to be working conditions. For the characterization of the biosensor some parameters such as reproducibility, thermal and pH stability were carried out.

1. Introduction

Tyrosinase, phenoloxidase and catecholoxidase are copper protein family. All of them have common active site but different functions. Tyrosinases use molecular oxygen to catalyse two different enzymatic reactions, (i) the ortho-hydroxylation of monophenols to o-diphenols (monophenolase, cresolase acticity) and (ii) the oxidation of o-diphenols to o-quinones (diphenolase, catecholase activity) [1,2]. Enzymes that can catalyze both reactions are sometimes referred to as tyrosinases (EC 1.14.18.1), while those that carry out only the latter reaction may be defined as catechol oxidases (EC 1.10.3.1) [3].

Catechol oxidase and tyrosinase initiate the synthesis of melanin and are responsible for pigmentation of skin and hair, browning of fruit and wound healing in plants and arthropods [4]. Phenol oxidases (PPOs) are widely dispersed among almost all organisms and taken part in several critical functions; they are generally known for their role in the browning of fruit or mushrooms. All type 3 copper proteins have a very similar active site but they have different structure and sequence [4–6]. It is made up by two histidine-coordinated copper atoms. In the oxy form one dioxygen molecule is bound in a side-on bridging coordination between the copper atoms. Each copper atom is bounded to the protein matrix by three histidine residues provided by an antiparallel α-helix pair. [7]. Almost all organisms have phenol oxidase that is satisfied several essential biological functions.[8–10].

The active site of all polyphenol oxidase is a dinuclear copper center consisting of two copper ions, each coordinated to three histidine side chains. This type-3 copper center binds molecular oxygen in a side-on bridging binding mode. Studies of model compounds continue to provide insight into the possible catalytic mechanism by which PPOs oxidize phenolic compounds. The common feature is a “type 3 copper centre”, a diamagnetic spin-coupled copper pair. Each of the two metal atoms, CuA and CuB, of the active site are coordinated by three conserved histidines which are located in a four a-helix bundle [10].

Tyrosinases, widely scattered among animals, plants and fungi and they are involved in many biologically essential functions such as pigmentation, sclerotization, primary immune response and host defence. Although tyrosinases and their inactive forms have been well characterized with respect to their sequences and enzymatic activity, only little is known about the native structure of the whole tyrosinase molecule [11]. Catechol oxidase (CO) is the designation for enzymes that catalyze only the latter reaction.

The total body content of copper is 150 mg. Approximately 30% is absorbed from the gastrointestinal tract. In blood, copper is initially albumin-bound and transported via the hepatic portal circulation to the liver [12].

In laboratory and industrial use, copper is an important element that is catalyst oxidation. Copper–dioxygen complexes are recommended to

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play important roles in these catalytic oxidation reactions, yet few such intermediates have been characterized, and the structural factors confirm the catalytic impressiveness of the copper catalysts remain uncertain. The oxidation chemistry of well-characterized copperdioxygen complexes is, thus, expected to provide the basis for designing and developing new catalytic oxidation systems with copper as a main catalytic component. Another primary interest in the copper–dioxygen complexes originate from the varied occurrence of copper proteins which function as highly efficient biooxidation catalysts. Copper is an plentiful element for proteins involved in dioxygen metabolism (exceeded probably only by iron), and a variety of physiological functions are known, dioxygen carrier, oxidase, oxygenase, and superoxide dismutase. Copper–dioxygen adducts are suggested as key reaction intermediates in these enzymatic reactions [13].

A biosensor can be used to monitor the presence of product, either biomass, enzyme, or antibody, or of a by-product of the process as an indirect measure of process conditions. Biosensors could make monitoring and control in the biotech industry accurate and reproducible. Specific fields of interest are the bioprocess industry, the medical field, environmental detection of pollutants or toxins [14–24]. Some original and attractive biosensors were developed for the determination of copper such as microbial [25–28], carbon paste based [29–32] and optical [33,34]. In this study tyrosinase based biosensor has been developed for the investigation of the positive effect of copper ions on the activity of the enzyme.

2. Experimental

2.1. Chemicals

Tyrosinase (monophenol monooxygenase) (E.C 1.14.18.1) from mushroom (*Agaricus bisporus*), calf skin gelatin, catechol, glutaraldehyde (25%, v/v), $CuSO_4.5H_2O$, KH_2PO_4, K_2HPO_4, $MgSO_4$, $MnCl_2$, $ZnCl_2$, $(NH_4)_2SO_4$ and all other chemicals were purchased from Sigma-Aldrich Chemical Co. (USA).

2.2. Apparatus

In the experiments, a YSI model 58 Digital Oxygenmeter, YSI 5700 model dissolved oxygen (DO) probes, Gilson P100 and P1000 automatic pipets (France), Pharmacia spectrophotometer (UK), Nuve model thermostat (TR), Yellow-Line magnetic stirrer (Germany), and high-sensitive teflon membranes (0.0005 in. thick) were used.

2.3. Construction of the biosensor

One of the most important step in the development of biosensors is the immobilization of the biological component at the transducer surface. The immobilization procures both the stabilization of the biomaterial and the proximity between the biomaterial and the transducer.

In the experiments dissolved oxygen probe(DO) was covered with a high-sensitive teflon membrane by using an O-ring and then teflon membrane which is selective and sensitive for oxygen, was pretreated with 0.5% SDS (sodiumdodecylsulfate) in phosphate buffer (50 mM, pH 7.0) to reduce the tension on the membrane surface of the probe. After that, tyrosinase (69 U/cm^2) and gelatin (4.21 mg/cm^2) were mixed in phosphate buffer (210 μl) at 38 °C for a few minutes to dissolve the mixture. In order to be formed a bioactive layer on the probe 200 μl of the solution was spread over the DO probe membrane surface and allowed to dry at 4 °C for 1 h and then the biosensor was crosslinked with glutaraldehyde (2.5%) in phosphate buffer (50 mM, pH 7.0) for 3 min.

2.4. Principle of the measurements

The working principle of the biosensor depends on the reaction given below.

$$\text{Catechol} + {}^{1}\!/_{2}O_2 \xrightarrow{\text{Tyrosinase}} \text{o} - \text{Quinone} + H_2O$$

The principle of the measurement is based on the determination of the differences in consumed dissolved oxygen concentration related to enzymatic reaction in the absence and the presence of copper. Copper ions showed an activator effect for the tyrosinase and injection of the copper solution into the reaction medium increases the biosensor responses. As a result, the differences in the consumed dissolved oxygen concentrations in the absence and the presence of copper ions were proportional to the concentrations of copper ions. When the substrate and copper ion were not in the reaction medium, there was no any enzymatic reaction in the medium and only a steady-state dissolved oxygen concentration was monitored. After addition of the catechol into the reaction medium a new dissolved oxygen concentration (DO_1) was obtained in the absence of copper ion. And then, the measurement was repeated for the same catechol standard in the presence of the copper ion and the dissolved oxygen concentratin (DO_2) was obtained. Differences between (DO_2) and (DO_1) gave us the positive effect of copper ion on the activity of tyrosinase $[(DO_2)-(DO_1)]-DO_3$. DO_3 value was related to the copper concentration added into the reaction medium. As a result, the differences in the dissolved oxygen values in the absence and the presence of copper were detected by the biosensor to obtain a standard curve for the determination of copper. All the measurements were done at 30 °C by using a thermostatic reaction cells and the oxygen saturated phosphate buffer (50 mM, pH 7.0).

3. Results and discussion

3.1. Optimization of the bioactive surface of the biosensor

3.1.1. Effect of the enzyme activity on the biosensor response

The effect of enzyme amount on the electrode response is shown in Fig. 1. The enzymatic activity of the bioactive membrane layer depended upon the amount of enzyme. To detect the effect of the enzyme activity on the biosensor response different enzyme amounts were used in the construction of the biosensor. Three biosensor which

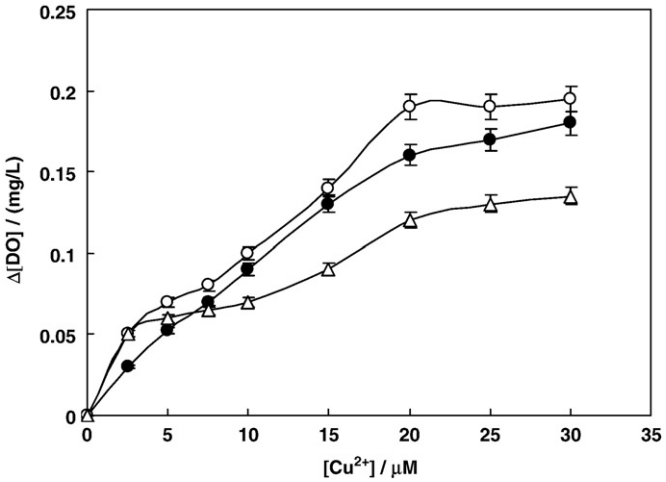

Fig. 1. The effect of tyrosinase activity on the biosensor response. (Phosphate buffer; pH 7.0, 50 mM; *T* 30 °C). The activity of tyrosinase; (○–○) 34.5 U/cm^2, (●–●) 69 U/cm^2, (Δ–Δ) 138 U/cm^2. The concentration of catechol, the percentage of glutaraldehyde and gelatine amount were kept constant to be 2.5 μM, 2.5% , 4.21 mg/cm^2, respectively.

contain 34.5, 69 and 138 U/cm^2 activity of tyrosinase were prepared by immobilizing with gelatin (4.21 mg/cm^2) and glutaraldehyde (2.5%). The measurements were made to obtain standard curves for copper by using the biosensors prepared (Fig. 1).

As illustrated in the figure, the most useful calibration curve was obtained by using the biosensor which was prepared with 69 U/cm^2 activity of tyrosinase. Copper was detected with a linear range between 2.5 and 20 μM concentrations by this biosensor. More higher biosensor responses were obtained when the tyrosinase activity was increased from 69 U/cm^2 to 138 U/cm^2 but the linearity was swerved. Decrease in the enzyme amount from 69 U/cm^2 to 34.5 U/cm^2 created lower biosensor responses and the negative effect for the linearity. From the results it can be said that, the most suitable biosensor responses were obtained by the biosensor contained 69 U/cm^2 activity.

3.1.2. Detection of the effect of gelatin amounts on the biosensor response

For the determination of the effect of gelatin amounts on the biosensor response different amounts of gelatin were used in the construction of the biosensors. Biosensors contained 2.11 mg/cm^2, 4.21 mg/cm^2 and 8.42 mg/cm^2 gelatin and 69 U/cm^2 tyrosinase. All the biosensors were immobilized by glutaraldehyde (2.5%). The measurements were made to obtain standard curves for copper by using the biosensors prepared (Fig. 2).

From the figure, decrease in the gelatin amount from 4.21 mg/cm^2 to 2.11 mg/cm^2 created lower biosensor responses and the negative effect for the linearity. The reason of this effect was the reducing in the forming of cross-linked bonds between enzyme–gelatin–glutaraldehyde. When the gelatin amount was increased from 4.21 mg/cm^2 to 8.42 mg/cm^2 increases were detected in the biosensor responses but the linearity was swerved. The reason of this effect can be arisen from the increasing in the forming of cross-linked bonds between the enzyme and gelatin. In this case, the substrate and activator can not diffuse toward to bioactive layer of the biosensor for a better interaction with the enzyme active center. The results obtained from the experiments showed that the most suitable biosensor responses were obtained by the biosensor prepared with 4.21 mg/cm^2 gelatin.

3.1.3. Effect of concentration of glutaraldehyde on the biosensor response

To detect the effect of the concentration of glutaraldehyde on the biosensor responses different amounts of glutaraldehyde were used in the construction of the biosensor. For this purpose we prepared biosensors, which contain 69 U/cm^2 tyrosinase and 4.21 mg/cm^2 gelatin. On the other hand the biosensors were treated with 1.25, 2.5, and 5.0% (v/v) glutaraldehyde solution prepared in phosphate buffer (50 mM, pH 7.0) for the immobilization. After immobilization procedure the experiments were made to obtain a standard curve for copper by using the biosensor prepared (Fig. 3).

From the experiments the best linear range and the reliable responses were obtained when 2.5% glutaraldehyde was used. At this concentration the responses of the biosensor were reproducible and the linear curve was fairly smooth. Lower concentration of glutaraldehyde was not sufficient enough to allow adequate cross-linking of the enzyme and gelatin and this could be a possible explanation for this result. When the percentage of glutaraldehyde was increased from 2.5% to 5%, decreases in the biosensor responses were observed and the linearity was swerved. This result probably due to the much more cross-linked bonds occured between the enzyme and gelatin.

3.2. Optimization of working conditions

3.2.1. pH effect on the biosensor response and pH stability

For the determination of the effect of pH value on the biosensor response different buffer systems were investigated. For this purpose 50 mM concentration of citrate (pH 5–6), phosphate (pH 7–8), and glycine (pH 9–10) buffers were used in the experiments. From the experiments the optimum pH value was obtained to be 7.0 (Fig. 4). Below and above this pH value decreases were observed on the biosensor responses. In the literature, optimum pH value of tyrosinase obtained from *Agaricus bisporus* shows a little variety. Munoz et al. [41] found it to be pH 7.0, Khan et al. [42] and Matsuura et al. [43] found it to be pH 6.8. Munoz et al. also reported that the pH range of tyrosinase is between 4.5 and 7. According to these data it can be said that the optimum pH value of free tyrosinase was not affected with the immobilization procedure used in the preparation of the biosensor.

pH stability is a very important factor for the enzyme and also enzyme based biosensors. This factor can effect the operational and also storage stability of biosensors. If the pH stability of a biosensor is optimum at the working pH this is the best and the preferencial condition. For the investigation of the pH stability of the biosensor some buffers such as citrate (pH 5.0 and 6.0), phosphate (pH 7.0 and 8.0), and glycine (pH 9.0 and 10.0) were used. The biosensor was kept in these buffers for one hour. At the end of the period the measurements were made for 2.5 μM catechol in the presence of 10 μM copper solution under the optimum conditions (pH 7.0, 50 mM phosphate buffer and 30 °C). If we consider the results obtained from

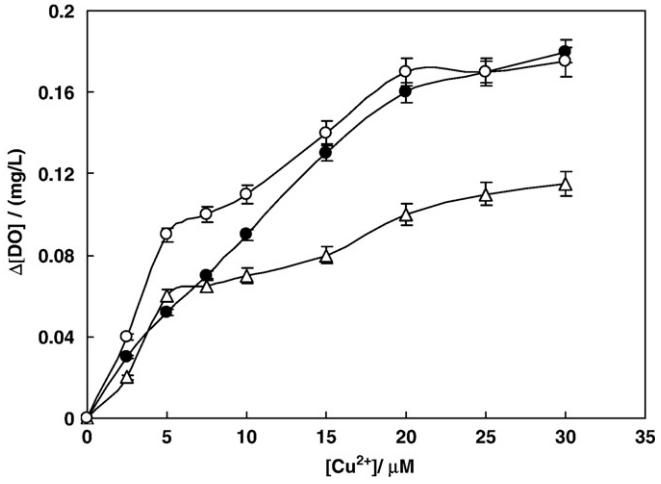

Fig. 2. The effect of gelatin amount on the biosensor response. Phosphate buffer; pH 7.0, 50 mM; T 30 °C; (Δ–Δ) 2.11 mg/cm^2; (●–●) 4.21 mg/cm^2; (○–○) 8.42 mg/cm^2. The activity of tyrosinase, the concentration of catechol, the percentage of glutaraldehyde were kept constant to be 69 U/cm^2, 2.5 μM, 2.5%, respectively.

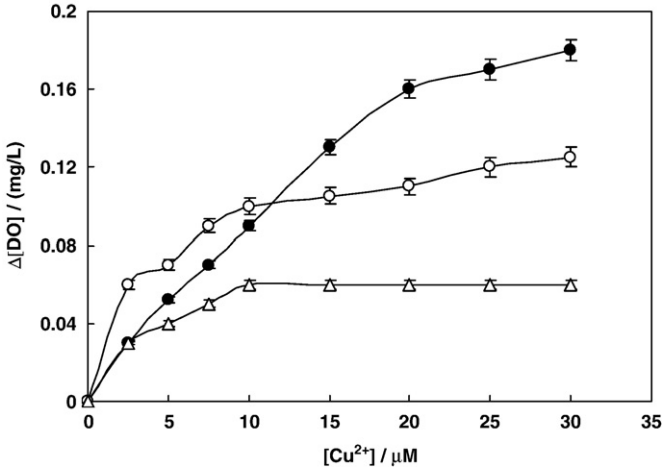

Fig. 3. The effect of concentration of glutaraldehyde on the biosensor response. (○–○) 1.25%, (●–●) 2.5%, (Δ–Δ) 5.0% glutaraldehyde. Phosphate buffer; pH 7.0, 50 mM; T 30 °C. The activity of tyrosinase, the concentration of catechol and gelatin amount were kept constant to be 69 U/cm^2, 2.5 μM, 4.21 mg/cm^2, respectively.

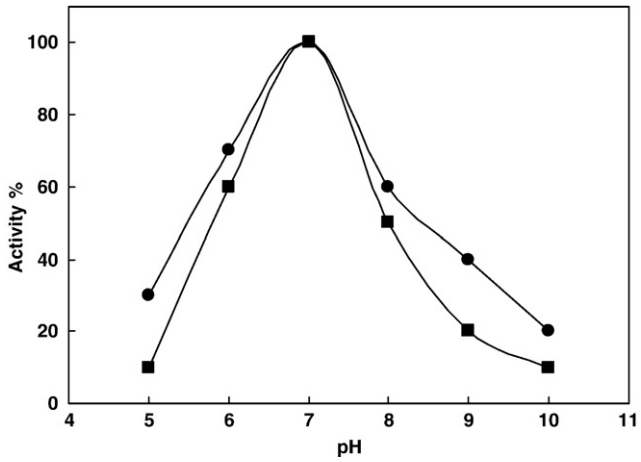

Fig. 4. The detection of the optimum pH and the pH stability of the biosensor. The concentration of all buffers is 50 mM. Citrate buffer (pH 5–6), phosphate buffer (pH 7–8), and glycine buffer (pH 9–10). $T = 30$ °C. (●–●) pH stability, (■–■) Optimum pH.

the measurements it can be said that the pH stability of the biosensor was optimum at pH 7.0. Fig. 4 shows the results obtained from the experiments.

3.2.2. Effect of temperature on the biosensor response

Because of the nature of the biocomponent used in the biosensor construction and also its relation with temperature, detection of the effect of the temperature on the biosensor responses is one of the most important factors for the biosensor studies. For the detection of temperature effect on the biosensor responses, the experiments were carried out between 15 and 45 °C. According to the results, the highest biosensor responses were observed at 30 °C. Below and above 30 °C, decreases in the biosensor responses were recorded. (Fig. 5). Results obtained from the experiments nearly corresponds with the studies about tyrosinase reported by Khan et al. [42], Matsuura et al. [43] and Gandia-Herrero et al. [44].

3.3. Analytical characteristics of the biosensor

3.3.1. Linear range of the biosensor

Standard curves for catechol obtained by the biosensor in the absence and the presence of copper ion (10 μM) are given in Fig. 6. As can be seen from the figure, the responses of the biosensor depend linearly on catechol concentration between 0.5 and 7.5 μM for both of the two curves obtained. Therefore, the biosensor responses were higher in the presence of copper ion because of the activation effect of copper ion.

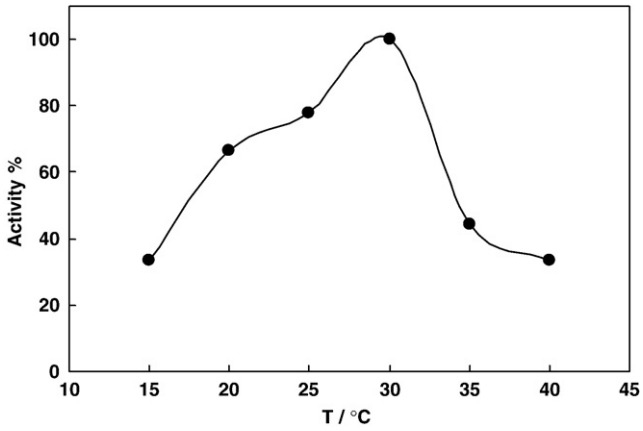

Fig. 5. The optimum temperature of the biosensor (Phosphate buffer; pH 7.0, 50 mM).

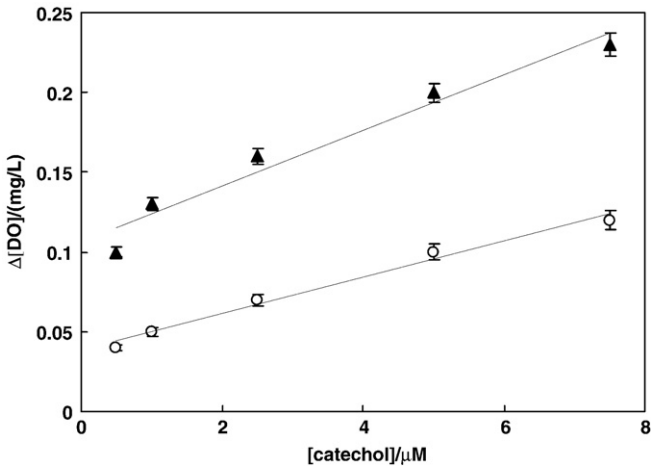

Fig. 6. The effect of copper ion on the biosensor response. (Phosphate buffer; pH 7.0, 50 mM.), (○–○) without copper, (▲–▲) with copper (10 μM). The activity of tyrosinase, the concentration of copper sulphate, the percentage of glutaraldehyde and gelatin amount were kept constant to be 69 U/cm^2, 10 μM, 2.5% and 4.21 mg/cm^2, respectively.

Measurements were made by the biosensor in order to obtain a standard curve for copper. For this purpose,first, a dissolved oxygen concentration was obtained (DO_1) for 2.5 μM catechol in the absence of copper ion. After that, different concentrations of copper (2.5–20 μM) were added into the reaction medium in the presence of the same constant concentration of catechol (2.5 μM) and new dissolved oxygen concentrations were obtained as (DO_2), (DO_3), (DO_4), etc. Differences between the obtained DO values and (DO_1) gave us a linear standard curve related to copper concentrations. Results obtained from the experiments are given in Fig. 7.

When we consider the figure, the biosensor responses depend linearly on copper concentration between 2.5 and 20 μM with ($y = 0.0071x + 0.0177$) and $R^2 = 0.9911$. The detection limit of the biosensor was calculated to be 0.95 μM by using $3S_b/m$ criteria, where m is the slope of the calibration curve and S_b is the standard deviation of the responses ($n = 10$) from 2.5 μM Cu^{2+}. Table 1 shows a comparison of analytical parameters such as the linear range and detection limit of some sensors and biosensors developed for copper assay [27,28] and [32–40]. The biosensor offers attractive properties compared to some of previous studies such as high sensitivity, a wide linear range (2.5 μM–20.0 μM), and low detection limit (0.95 μM).

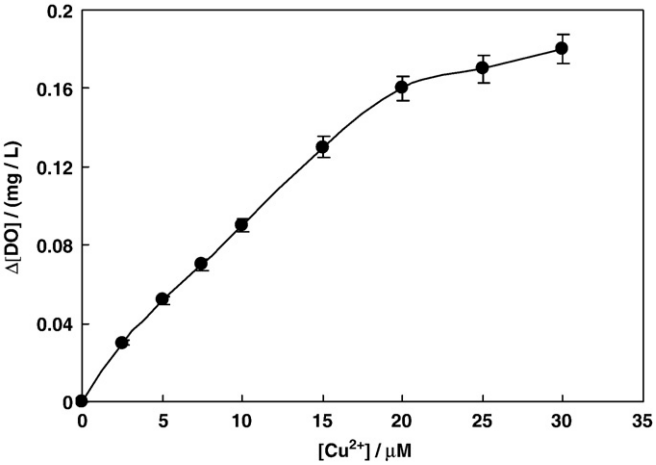

Fig. 7. Standard curve of the biosensor for copper. (Phosphate buffer; pH 7.0, 50 mM; T 30 °C.) The activity of tyrosinase, the concentration of catechol, the percentage of glutaraldehyde and amount of gelatin were kept constant to be 69 U/cm^2, 2.5 μM, 2.5%, 4.21 mg/cm^2, respectively.

Table 1
Analytical parameters of some sensors and biosensors for the determination of Cu^{2+}.

Sensor or biosensor type	Linear range	Detection Limit	Reference
Reflection-based optic fiber sensor	0.01–1.0 μg/ml	5.0 ng/ml	[33]
Chemiluminescence	0.01–10 μg/L	8.0×0^{-3} μg/L	[34]
Fluorescence	0.039–14.0 μM	0.039 μM	[35]
Chelating ionophores	5.0×10^{-8}–1.0×10^{-2} M	4.0×10^{-8} M	[36]
Glucose oxidase based	0.0125–0.1 μg/L	0.0125 μg/L	[37]
Anodic stripping voltammetry	8.0×10^{-6}–8.0×10^{-5} M	4.0×10^{-7} M	[38]
Tobramycin-nafion modified electrode	1.0×10^{-9}–5.0×10^{-7} M	5.0×10^{-10} M	[39]
Microbial based on *Circinella* sp.	5.0×10^{-7}–1.0×10^{-5} M	5.4×10^{-8} M	[27]
Microbial based on *Tetraselmis chuii*	5.0×10^{-8}–1.0×10^{-6} M	4.6×10^{-10} M	[28]
CPE containing a natural zeolite	5.0×10^{-8}–1.0×10^{-6} M	1.5×10^{-8} M	[32]
Enzyme thermistor	5.0×10^{-6}–1.0×10^{-4} M	5.0×10^{-6} M	[40]
This biosensor	0–20.0×10^{-6} M	0.95×10^{-6} M	

3.3.2. Reproducibility

To detect the reproducibility of the biosensor some experiments were investigated for 10 μM concentration of copper sulphate ($n=10$). From the experiments, the average value ($\bar{x}$), standard deviation (SD) and coefficient of variation (CV %) of the biosensor were calculated to be 9.95 μM, ±0.23 μM and 2.33%, respectively.

3.3.3. Metal ion specificity

In order to detect the metal ion specificity of the enzyme different metal ions which has +3, +2 and +1 charged were used. For this purpose, experiments were made by using 2.5 μM catechol, 5,10 and 20 μM concentrations of copper and various metal ions such as Mn^{2+}, Mg^{2+}, $(NH_4)^+$, Zn^{2+} and Fe^{3+}. The biosensor response obtained for copper was accepted as 100% and this result was compared to other biosensor responses obtained for these metal ions. Also to detect the effect of tested metal ions in the presence of Cu^{2+} some experiments were done by the biosensor. For this purpose the metal ions and Cu^{2+} that are at the same concentration, were added together into the reaction medium. Results obtained from the experiments were given in Table 2. Data given in the Table are average of five measurements.

According to the results, when 5 and 10 μM concentrations of Fe^{3+}, Mn^{2+}, Mg^{2+}, $(NH_4)^+$ and Zn^{2+} were used in the experiments there was no any activator effect for the tyrosinase. But at 20 μM concentrations of the metal ions we observed that only Mn^{2+}, Mg^{2+} and Zn^{2+} ions increased the activity of the enzyme. The percentages of the activation effect of Mn^{2+}, Mg^{2+} and Zn^{2+} ions were detected to be 8%, 13%, and 11%, respectively. These positive effects of Mn^{2+}, Mg^{2+} and Zn^{2+} ions showed a little decreases in the presence of Cu^{2+} ion. In this case it can be said that, copper ion has a better effect on the the activity of tyrosinase enzyme and the activation effect depends on the type and the concentration of the metals.

Table 2
Effect of metal ions on the biosensor response in the absence and the presence of Cu^{2+} ion.

Metal ion[a]	Activity % (for 5.0 μM)	Activity % (for 10.0 μM)	Activity % (for 20.0 μM)
Cu^{2+}	100	100	100
Mn^{2+}	0	0	8.0
(NH_4^+)	0	0	0
Mg^{2+}	0	0	13.0
Fe^{3+}	0	0	0
Zn^{2+}	0	0	11.0
$(Cu^{2+}+Mn^{2+})$	100	100	105
$(Cu^{2+}+(NH_4^+))$	100	100	100
$(Cu^{2+}+Mg^{2+})$	100	100	109
$(Cu^{2+}+Fe^{3+})$	100	100	100
$(Cu^{2+}+Zn^{2+})$	100	100	106

[a] Average of five measurements.

4. Conclusion

In this study, a novel, sensitive, simple and not-consuming biosensor system was developed for copper determination. The principle of the determination was based on the positive effect of copper on the activity of tyrosinase. The biosensor offers attractive properties such as reproducibility, high pH stability, response time and also metal ion specificity. Compared with the existing tyrosinase biosensor for determination of copper, our biosensor has significant differences in the preparation of the biosensor and also the determination method. Lu et al. developed a new system for the amperometric detection of Cu^{2+} using a reflection-based optic fiber sensor associated with flow-injection analysis. The sensing layer consists of Nitroso-R salt immobilized on Amberlyst A-27 resin. The sensor was not fully reversible but can be fully regenerated by using 1.0 M hydrochloric acid after each measurement. [33]. Qin et al. developed a novel chemiluminescence (CL) flow-through sensor based on immobilizing all the ingredients involved in the analytical reaction for the determination of copper. The analytical reagents including luminol and cyanide were coimmobilized permanently on an anion-exchange column. The sensor was not susceptible to interference by other metal ions associated with the chemiluminescence reaction [34]. All the chemical and also equipments used in the experiments are very expensive. From the literature it can be said that there is no any biosensor study that used tyrosinase enzyme and also our measurement method for the determination of copper.

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