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An inhibition type amperometric biosensor based on tyrosinase enzyme for fluoride determination

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ABSTRACT

In this study, a new biosensor based on the inhibition of tyrosinase for the determination of fluoride is described. To construct the biosensor tyrosinase was immobilized by using gelatine and cross-linking agent glutaraldehyde on a Clark type dissolved oxygen (DO) probe covered with a teflon membrane which is sensitive for oxygen. The phosphate buffer (50 mM, pH 7.0) at 30 °C were established as providing the optimum working conditions. The method is based on the measurement of the decreasing of dissolved oxygen level of the interval surface that related to fluoride concentration added into reaction medium in the presence of catechol. Inhibitor effect of fluoride results in decrease in dissolved oxygen concentration. The biosensor response depends linearly on fluoride concentration between 1.0 and 20 μM with a response time of 3 min.

In the characterization studies of the biosensor some parameters such as reproducibility, substrate specificity and storage stability were carried out. From the experiments, the average value (x), Standard deviation (S.D) and coefficient of variation (C.V %) were found as 10.5 μM, ± 0.57 μM, 5.43%, respectively for 10 μM fluoride standard.

1. Introduction

Tyrosinase is found in almost all organisms and catalyses several essential biological reactions. It initiates the synthesis of melanin and is responsible for pigmentation of skin and hair, browning of fruit and wound healing in plants and arthropods [1]. Tyrosinase is a binuclear copper containing metalloprotein that oxidizes monophenols and *o*-diphenols into their corresponding *o*-quinones, at the expense of oxygen reduction to water. The conversion of monophenols by tyrosinase proceeds in two consecutive steps, i.e. in the first step monophenol is hydroxylated to its corresponding *o*-diphenol (hydroxylase activity), which in a second step is oxidized to its corresponding *o*-quinone, whereby the enzyme is oxidized by molecular oxygen back to its native form (the enzyme's catecholase activity) [2]. This enzyme which produced from mushrooms has an isoelectric point of 4.5 and carries a negative charge at pH >4.5 in aqueous solution [3]. Tyrosinase tightly inhibits by copper chelators (competitive with respect to oxygen) like tropolon, benzhydroxamic, salicylhydroxamic acid. In addition halide anions such as fluoride, chloride, iodide and bromide play a role in the inhibition of tyrosinase. The fluoride and chloride inhibitions appear competitive, whereas iodide and bromide inhibit through an apparent non-competitive mechanism. The order of strength of inhibition is I > F > Cl > Br with apparent inhibition constants of 3.8 mM, 11 mM, 0.16 M, and 0.23 M, respectively [4–10].

Sodium fluoride is an ionic compound with the formula NaF. This colourless solid is the main source of the fluoride ion in diverse applications. Fluoride salts are used widely to enhance the strength of teeth by the formation of fluoroapatite, a naturally occurring component of tooth enamel. Toothpaste often contains sodium fluoride to prevent cavities. Sodium fluoride is also used as an antibiotic, as rat poison, and in ceramics. Sodium fluoride strongly reduces tyrosinase activity like acid and cyanide and also inhibition of sodium fluoride is highly depending on pH [11–13].

Biosensors, one of newer areas in analytical chemistry, have simple analysis methods, reproducibility, non-expensive, sensitivity and short response time. Fluoride can be accurately detected by using fluorescence systems [14], immunoassay methods [15], potentiometric chemical sensors [16], colorimetric methods [17–18], spectrophotometry [19], ion chromatography [20], high-performance liquid chromatography [21], optical sensors [22] and gas chromatography [23]. Although all these methods are currently available for fluoride detection, some of them present complicated and time-consuming protocols and also they required expensive equipment and advanced technical support especially to comment on the problems about the results and equipments.

In this study we have developed a tyrosinase biosensor for the detection of fluoride by using its inhibitory effect on the activity of tyrosinase. This effect was monitored amperometrically by

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using Clark type dissolved oxygen electrode that is combined YSI oxygenmeter.

2. Experimental

2.1. Chemicals

Tyrosinase (E.C 1.14.18.1) from mushroom (*Agaricus bisporus*) and calf skin gelatine (225 bloom), glutaraldehyde (25%), sodium fluoride, catechol and all other chemicals were purchased from Sigma Chemical Co. (USA). All solutions used in experiments were prepared just before their use.

2.2. Apparatus

In the study, a YSI Model 58 digital dissolved oxygen (DO) oxygenmeter, YSI 5739 Model DO probes (with YSI 5740 model cable)(YSI Co., Yellow Springs, USA), high sensitive teflon membranes (0.0005 in. thick) for oxygen and Nuve Thermostat (Ankara, TR) were used for constant temperature in the experiments.

2.3. Preparation of the biosensors

First DO probe was covered with standard teflon membrane by using an O-ring and then teflon membrane which is selective for oxygen was pre-treatment with 0.5% SDS (sodiumdodecylsulfate) in phosphate buffer (50 mM, pH 7.5) to reduce the tension on the membrane surface, thus microbial contamination was prevented. After that, tyrosinase (69 U), and gelatine (5 mg) were mixed in 300 μl of phosphate buffer (pH 7.0; 50 mM) for a few minutes. The mixture was dissolved at 38 °C. 200 μl of the solution was spread over the DO probe membrane surface and allowed to dry at 4 °C for 25 min and then the enzyme was cross-linked with glutaraldehyde solution (2.5%) in phosphate buffer (50 mM, pH 7.5) for 4 min. Excess of glutaraldehyde was eliminated by washing phosphate buffer and double distilled water.

2.4. Principle of the measurement

The principle of the measurement is based on the determination of decreasing oxygen level by the activity of tyrosinase and the detection of changes in consumed oxygen level related to concentration of fluoride added into the reaction medium.

$$\text{Catechol} + \tfrac{1}{2}O_2 \xrightarrow{\text{Tyrosinase}} \text{Benzoquinone} + H_2O$$

At the beginning, when there is no fluoride in the reaction medium, consumed oxygen level obtained from the enzymatic reaction is measured as (DO_I). After that, fluoride is injected into the reaction medium together with catechol and decrease in the consumed oxygen level is determined as (DO_{II}). Difference between two measurements ($DO_I - DO_{II} = DO_{III}$) is related to concentration of fluoride added into the reaction medium. By using this inhibition based biosensor we detected the both substrate (catechol) and inhibitor (fluoride) of enzyme.

All the measurements were carried out at 35 °C by using thermostatic reaction cells and oxygen saturated phosphate buffer (50 mM, pH 7.0).

3. Results and discussion

3.1. Detection of the inhibition effect of fluoride on the activity of tyrosinase

For the detection of the inhibition effect of fluoride on the activity of tyrosinase biosensor some experiments were made. In experiments first, the biosensor was used for obtaining a standard curve for catechol detection by using catechol standards between 0.5 μM and 7.5 μM concentration in the absence of fluoride. In the second step, by using the same catechol standards, that all of them containing 10.0 μM fluoride, a new standard curve was obtained. From the second standard curve, we investigated that fluoride competitively decreased the biosensor responses. Fig. 1 shows this obvious effect on the tyrosinase enzyme.

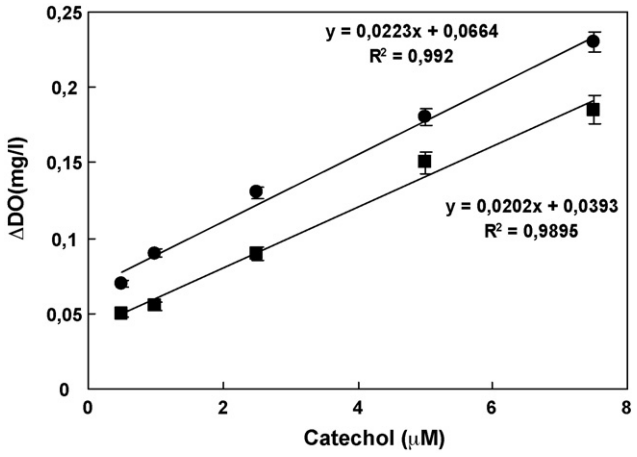

Fig. 1. The inhibition effect of sodium fluoride on the biosensor response (phosphate buffer; pH 7.0, 50 mM at 30 °C). (●) without fluoride; (■- with fluoride. The activity of tyrosinase, the concentration of fluoride, the percentage of glutaraldehyde and gelatine amount were kept constant at 40.7 U/cm², 10 μM, 2.5%, and 4.21 mg/cm², respectively.

3.2. Optimization of the immobilization parameters

3.2.1. Effect of enzyme activity on the biosensor response

To determine the effect of enzyme activity on the biosensor response, different amounts of tyrosinase were studied. For this purpose, we prepared three different biosensors that contained 20.35 U/cm², 40.7 U/cm² and 81.4 U/cm² activity of tyrosinase, and all of them immobilized with gelatine (5 mg) and glutaraldehyde (2.5%) as cross-linking agent. Results obtained from the experiments were shown in Fig. 2. The best useful curve was obtained by the biosensor which was prepared to 40.7 U/cm² activity of

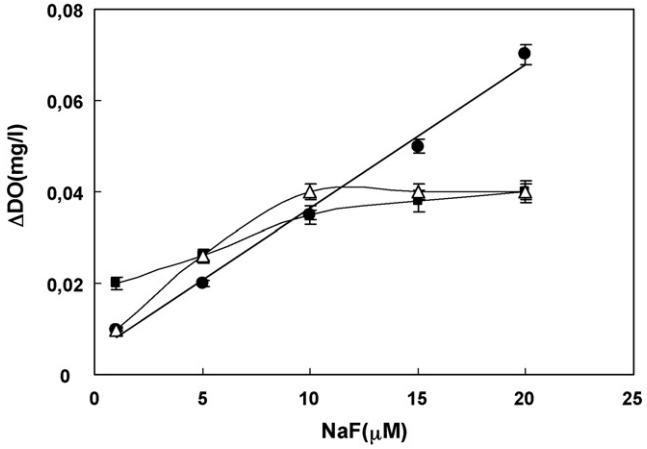

Fig. 2. The effect of tyrosinase activity on the biosensor response. The activity of tyrosinase; (Δ-Δ) 20.35 U/cm², (●) 40.7 U/cm², (■- 81.4 U/cm². Phosphate buffer; pH 7.0, 50 mM; T = 30 °C; the concentration of catechol, the amount of gelatine and the percentage of glutaraldehyde were kept constant at 2.5 μM, 4.21 mg/cm², 2.5%,respectively.

tyrosinase. Response of this biosensor was higher and correlated linearly at fluoride concentration between 1 μM and 20 μM. By using the other biosensors containing 20.35 U/cm^2 and 81.4 U/cm^2 tyrosinase activity, narrow linear regions that were correlated linearly at fluoride concentration between 1 μM and 10 μM were obtained. As a result, the best responses and linear range were obtained by the biosensor prepared to 40.7 U/cm^2 activity of tyrosinase.

3.2.2. Effect of gelatine amounts on the biosensor response

To determine the effect of gelatine amounts on the biosensor response different amounts of gelatine were used. For this purpose the biosensors containing 2.10 mg/cm^2, 4.21 mg/cm^2, and 8.42 mg/cm^2 gelatine, were prepared and used. All biosensors had 40.7 U/cm^2 tyrosinase activity and all of them were cross-linked by glutaraldehyde (2.5%). According to results obtained from experiments it can be said that fluoride was correlated linearly with a range between 1 μM and 20 μM concentrations by the biosensor prepared to 4.21 mg/cm^2 gelatine.

On decreasing the amount of gelatine from 4.21 mg/cm^2 to 2.10 mg/cm^2 showed a swerve on the curve and also decreases on biosensor responses. The most important reason of this effect was the escape of enzyme from the bioactive layer, due to insufficient tightness of the enzyme immobilization. When we used 8.42 mg/cm^{-2} gelatine in the biosensor preparation, the biosensor responses were very low and in addition we obtained a linear standard graph for the fluoride concentration only between 5 μM and 15 μM. Because, increasing of gelatine leads to thicker diffusion barriers in between the bioactive layer and the electrode surface. Thicker diffusion barrier hinders the effective working of the enzyme and also diffusion of substrate and inhibitor from the bioactive layer to interval surface. These experiments showed that optimal biosensor responses were achieved by the biosensor which contained 4.21 mg/cm^2 gelatine.

3.2.3. Effect of the percentage of glutaraldehyde on the biosensor response

In this part of the optimization studies, different percentages of glutaraldehyde were used in the biosensor preparation for the determination of the effect of the glutaraldehyde on the biosensor response. For this purpose first three biosensors containing 40.7 U/cm^2 activity of tyrosinase and 4.21 mg/cm^2 gelatine were prepared, and then the biosensors prepared were treated with 1.25%, 2.50%, and 5.0% glutaraldehyde solutions for cross-linking. When we used 1.25% concentration of glutaraldehyde in the biosensor preparation we detect a linear region that is between 1 μM and 10 μM concentrations of fluoride. In this concentration range the biosensor's responses higher than the other biosensor's responses which obtained by preparing 2.5% and 5.0% glutaraldehyde. Nevertheless, response obtained for 1 μM NaF was nearly the same, the response obtained for 10 μM. In this case, the linear region obtained was nearly horizontal and not useful for quantitative analysis. Lower concentrations of glutaraldehyde were not sufficient enough to allow adequate cross-linking of the enzyme and gelatine molecules .So, the most useful and better responses were obtained by using the biosensor prepared to 2.5% glutaraldehyde. From experiments it is obvious that fluoride was detected with a linear range between 1 μM and 20 μM concentrations by the biosensor which was prepared to 2.5%.

When we considered about this findings it is clear that an increase in the percentage of glutaraldehyde from 2.5% to 5.0% brought about an explicit decrease in biosensor responses. The most important reason of this effect was the formation of stricter cross-links. Hence catechol, the substrate molecule, could not be approached easily and converted into the product.

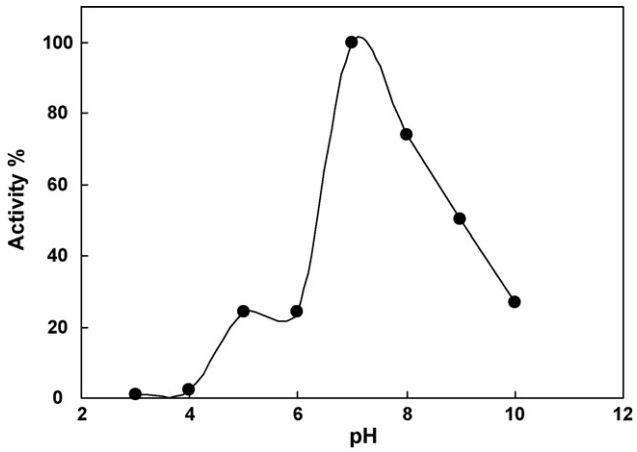

Fig. 3. The effect of pH on the biosensor response acetate buffer (3.0–4.0), citrate buffer (5.0–6.0), phosphate buffer (7.0–8.0) and glycine buffer (9.0–10.0) 50 mM, 30 °C). The activity of tyrosinase, the concentration of catechol and fluoride, the percentage of glutaraldehyde and amount of gelatine were kept constant at 40.7 U/cm^2, 2.5 μM, 10 μM and 4.21 mg/cm^2, respectively.

3.3. Optimization of working conditions

3.3.1. Effect of temperature on the biosensor response

To determine the effect of temperature on the biosensor response is very important due to nature of biocomponent and also obtain the best biosensor responses Experiments were carried out between 15 °C and 40 °C. From results, it can be said that the highest biosensor responses were obtained at 30 °C for biosensor. At upper and lower temperature than 30 °C decreases in the biosensor responses were observed.

3.3.2. Effect of pH on the biosensor response

The experiments were carried out at different buffer systems for the determination of the effect of pH value on the biosensor response. For this purpose 50 mM concentrations of acetate (pH 3.0–4.0), citrate (pH 5.0–6.0), phosphate (pH 7.0–8.0) and glycine (pH 9.0–10.0) buffers were prepared and used. Results obtained from experiments were shown in Fig. 3. According to findings, optimum pH value was achieved as 7.0. There was no response in the biosensor at pH 3.0 and pH 4.0 values. If we consider the optimum pH value (pH 7.0) of the enzyme it can be said that the most important factor in this result is the instability of the enzyme at pH values lower than ca. 5. In addition, from the literature Tepper et al. [5] were discussed that acidic form of the enzyme is capable for binding of fluoride.

3.4. Analytical characteristics of the biosensor

3.4.1. Linear range of the biosensor

After optimizations of the biosensor, experiments were carried out to achieve a calibration curve for fluoride at optimal conditions. From results it can be said that the biosensor responses correlated linearly at sodium fluoride concentration between 1.0 μM and 20 μM. From the experiments a linear regression equation was obtained for ($y = 0.0031x + 0.005$) with $R^2 = 0.9934$, where y represents ΔDO (mg/l) and x represents concentration of NaF (μM).

3.4.2. Reproducibility

For determination of the reproducibility of the biosensor experiments were also carried out at 10 μM sodium fluoride and 2.5 μM catechol concentration ($n = 8$). From experiments, the average value (x), standard deviation (S.D) and coefficient of variation (CV %) were calculated as 10.5 μM, ± 0.57 μM and 5.43% respectively.

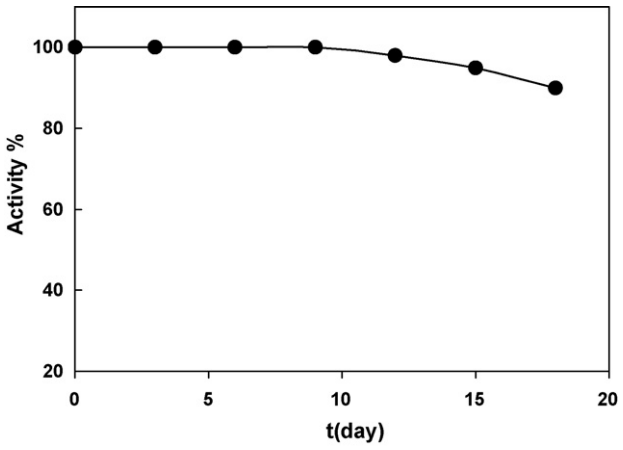

Fig. 4. Storage stability. (Phosphate buffer; pH 7.0, 50 mM at 30 °C) The activity of tyrosinase, the concentration of catechol and fluoride, the percentage of glutaraldehyde and amount of gelatine were kept constant at 40.7 U/cm^2, 2.5 μM, 10 μM, 2.5% and 4.21 mg/cm^2, respectively.

3.4.3. Storage stability

To observe storage stability of the biosensor a newly prepared biosensor was stored at +4 °C. Measurements were carried out in every third day along 18 days. The biosensor was used only for this experiment. Results obtained from experiments were shown in Fig. 4. Although 18 days were passed, there was only 10% loss in the activity and also responses of the biosensor for fluoride.

4. Conclusions

Inhibition based biosensors have been used very efficiently especially in environmental analysis. The principle of operation of these biosensors is based on the interaction that occurs between specific chemical and biological agents "inhibitors", present in the sample, and the biocatalyst such as an enzyme, a polyenzymatic sequence and/or even a whole tissue immobilized on the biosensor itself. The response of the biosensor is therefore proportional to the reduction rate of the enzymatic reaction which takes place at the sensor's interface.

This work demonstrates that the biosensor which is based on inhibition of fluoride has good analytical properties such as fast response (3 min), long-time stability and a good detection range and it is suitable for routine analysis of catechol and sodium fluoride at the same time. As a result, all of these advantages suggested that sodium fluoride can be detected easily, accurately and rapidly.

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