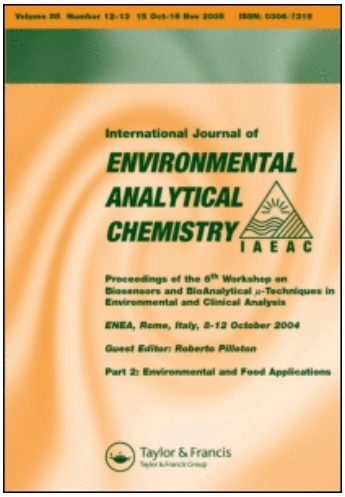

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Investigation of metal activation of a partially purified polyphenol oxidase enzyme electrode

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Investigation of metal activation of a partially purified polyphenol oxidase enzyme electrode

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Biosensors can be developed using different biological materials and immobilization technologies. Enzymes are generally used in biosensor construction, and some enzymes need metal ions or small organic molecules as a cofactor for their activation. Polyphenol oxidases can be activated by several metal ions such as Cu^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, and Ni^{2+}. In this study, a new measurement method has been developed that is based on the metal ion activation of the polyphenol oxidase enzyme used in the biosensor preparation, especially to determine the concentration of Mg^{2+} ions. Polyphenol oxidase (PPO) (EC 1.10.3.1) was partially purified from potato (*Solanum tuberosum*) by using $(NH_4)_2SO_4$ precipitation, dialysis, and lyophylization processes. As a result of this processes, approximately 30-fold purification was achieved for PPO. For construction of the biosensor, the enzyme was immobilized on the dissolved oxygen probe membrane using gelatin and glutaraldehyde (2.5%). Using the biosensor, we obtained responses for catechol in the absence and presence of Mg^{2+} ions. Differences between the biosensor responses were related to the concentration of Mg^{2+} ions. The biosensor response depends linearly on concentration of Mg^{2+} ions between 0.05 and 7.5 mM. In the optimization studies, phosphate buffer (pH 7.0, 50 mM) and 35°C were determined to be the optimum conditions. This project will be a novel biosensor study and it might bring a new term, 'activation based biosensor' into the biosensor area.

Keywords: Polyphenol oxidase; Biosensor; Activation-based biosensor; Mg^{2+} ions; Catechol

1. Introduction

Magnesium is an essential mineral mainly found in foods like cereals, nuts, cacao, meat, milk, water, mineral water, and vegetables. Magnesium has several important functions in the metabolism of the cells. It is involved in energy metabolism, acting as a metal activator or cofactor for enzymes requiring adenosine triphosphate (ATP), in replication of DNA, and in the synthesis of RNA and proteins; it appears to be essential for all phosphate-transferring systems. Magnesium is also involved, together with calcium ions, in muscle contraction and blood clotting. Magnesium, the second most abundant intracellular cation, has been identified as a cofactor in over

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300 enzymatic reactions involving energy metabolism and protein and nucleic acid synthesis [1, 2].

Because of the importance of magnesium, a great variety of methods can be used for magnesium analysis in different sample matrices such as spectrophotometric [3–6] fluorometric [7], liquid chromatographic [8], and sensoric [9–11] methods.

Polyphenol oxidase (PPO) [monophenol, dihydroxy-L-phenylalanine oxygen oxidoreductase, EC (1.10.3.1)] is a copper protein widely distributed on the phylogenic scale and responsible for the undesirable browning reactions during handling, storage, and processing of damaged tissues of fresh fruits and vegetables, as well as some animal products [12]. PPO catalyses two types of oxidative reactions: hydroxylation of monophenols to *o*-diphenols(cresolase activity) and oxidation to *o*-quinones (catecholase activity). PPO characteristics have been widely studied in various plants such as grapes [13], yam tubers [14], banana [15], plums [16], potato [17], tea [18], papaya [19], chickpea [20], and peaches [21].

The effects of some metal ions on polyphenol oxidase enzyme activity have been previously studied [22–24]. According to these studies, some metal ions such as Cu^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, and Mg^{2+} show an activator effect for the enzyme. Therefore, using these data, we have developed a partially purified polyphenol oxidase biosensor and investigated metal activation on the biosensor. Increases in the biosensor response were related to the concentrations of metal ions, and this relation has given us a linear graph to determine the metal concentration.

This project will be a novel biosensor study, and it will probably bring a new alternative point of view into the biosensor area and also metal-determination methods.

2. Experimental

2.1 *Chemicals and apparatus*

PPO (EC 1.10.3.1) was partially purified from potato (*Solanum tuberosum*) by $(NH_4)_2SO_4$ precipitation, dialysis, and lyophylization processes. Calf skin gelatin (225 bloom), glutaraldehyde (25%), catechol, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All solutions used in the experiments were prepared just before use. In the study, a YSI Model 58 digital dissolved oxygen (DO) oxygen meter, YSI 5739 Model DO probes (with YSI 5740 model cable) (YSI Co., Yellow Springs, OH), highly sensitive Teflon membranes (0.00127 cm thick) for oxygen, and a thermostat (Nüve, TR) for constant temperature were used in the experiments.

2.2 *Partial purification of PPO*

Peeled potato was homogenized in 150 mL of 0.1 M NaF. The homogenate was filtered through two layers of cheesecloth, and then the filtered material was centrifuged at 10,000 *g* for 15 min at 4°C. Solid $(NH_4)_2SO_4$ was added to the supernatant to yield 70% saturation, and then the mixture was centrifuged at 10,000 *g* for 15 min at 4°C. The precipitate was dissolved in 0.05 M phosphate buffer (pH 6.5). The enzyme extract was extensively dialysed against the same buffer at 4°C overnight and then lyophilized.

Lyophilized material was used as the PPO enzyme source in the following experiments. Protein was assayed by the Bradford method [25].

2.3 *Principle of the measurement*

According to the enzymatic reaction given below, the working principle of the biosensor is based on the determination of decreasing dissolved oxygen level, which is related to catechol concentration added to the reaction medium, and to the degree of activation of PPO by Mg^{2+} ions. Upon addition of Mg^{2+} ions, activation of PPO is observed.

$$2\text{Catechol} \xrightarrow{\text{PPO}} 2\ \text{1,2-Benzoquinone} + 2H_2O.$$

The principle of the Mg^{2+} ions determination by the biosensor is given below. First of all, using the biosensor, we obtained a standard curve for catechol concentrations (0.05–10.0 μM) in the absence of Mg^{2+} ions. Then, we obtained a new standard curve for the same catechol concentrations in the presence of a constant concentration of Mg^{2+} ions (2.5 mM). The biosensor responses of the second curve were higher than the first, and these increases resulted from the activation effect by Mg^{2+} ions of the PPO enzyme. After obtaining this result, by using different concentrations of Mg^{2+} ions (0.05–10.0 mM), we obtained a standard curve for Mg^{2+} ions with the biosensor in the presence of a constant concentration of catechol (2.5 μM). We observed that when the concentration of Mg^{2+} ions is increased from 0.05 to 7.5 mM in the presence of a constant concentration of catechol (2.5 μM), the activation effect by Mg^{2+} ions of the PPO enzyme occurred in a linear fashion.

As a result, using this activation-based biosensor, we can detect both the substrate of the enzyme (catechol) and the activator of the enzyme (Mg^{2+} ions).

In this study, $Cu^{2+}$and Mg^{2+} ions increased the responses of the PPO biosensor, but experiments focused only on effects of Mg^{2+} ions.

2.4 *Preparation of the biosensors*

First, a DO probe was covered with a highly sensitive Teflon membrane using an O-ring, and then the Teflon membrane, which is selective for oxygen, was pretreated with 0.5% sodium dodecylsulfate in phosphate buffer (50 mM, pH 7.0) to reduce the tension on the membrane surface. Then, PPO (1.87 U mg^{-1} solid) and gelatin (10 mg) were mixed in the phosphate buffer (300 μL) at 38°C for a few minutes to dissolve the mixture. Two hundred microlitres of the solution was spread over the DO probe membrane surface and allowed to dry at 4°C for 25 min, and then the biosensor was cross-linked with glutaraldehyde solution (2.5%) in phosphate buffer (50 mM, pH 7.0) for 4 min. At the end of this process, a bioactive layer was formed on the DO probe membrane of the biosensor.

3. Results and discussion

3.1 *Analytical characteristics of the biosensors*

PPO (EC 1.10.1.3) was partially purified from potato (*Solanum tuberosum*) by $(NH_4)_2SO_4$ precipitation and dialysis. Approximately 30-fold purification was achieved.

3.1.1 Linear range of the biosensors. Standard curves for catechol obtained by the biosensor in the absence and the presence of Mg^{2+} ions (2.5 mM) are given in figure 1. 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 Mg^{2+} ions than in the absence of Mg^{2+} ions.

For the different catechol concentrations, if the reaction medium contained a constant concentration of Mg^{2+} ions, the activity of PPO increased 24% or 100%.

For the determination of the concentration of Mg^{2+} ions and also for obtaining a standard curve of Mg^{2+} concentration measured by the biosensor, a DO concentration in 2.5 μM catechol in the absence of Mg^{2+} ions was obtained (DO_1). Then, different concentrations of Mg^{2+} ions (0.5–10.0 mM) were added into the reaction medium in the presence of the same constant concentration of catechol (2.5 μM), and new DO concentrations were obtained as (DO_2), (DO_3), (DO_4), etc., related to each of the Mg^{2+} ion concentrations added. The differences between (DO_2), (DO_3), (DO_4), etc. obtained and DO_1 gave us the biosensor responses related to Mg^{2+} ion concentrations. Results obtained from the experiments are given in figure 2. From this figure, using the biosensor, we can determine the Mg^{2+} ion concentration linearly between 0.5 and 7.5 mM.

3.2 *Optimization of the working conditions of the biosensor*

3.2.1 Effect of pH on the biosensor response. For the determination of the effect of the pH on the biosensor responses, different buffer systems were investigated for catechol (2.5 μM) in the presence of Mg^{2+} ions (2.5 mM). For this purpose, a 50 mM concentration of citrate (pH 5.0–6.0), phosphate (pH 7.0–8.0), and glycine (pH 9.0–10.0) buffers was used. The results obtained from these experiments are given in figure 3. According to the figure, the optimum pH of the biosensor was determined

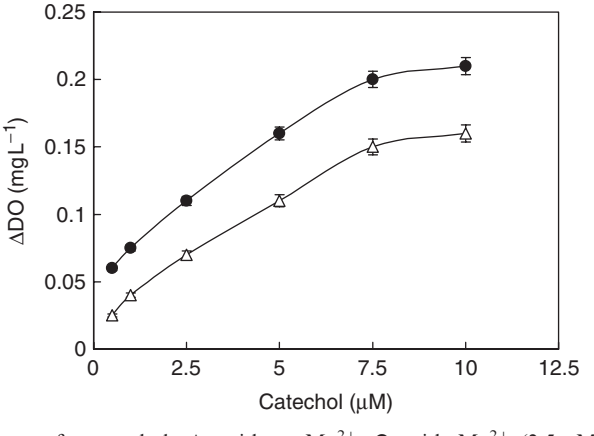

Figure 1. Standard curve for catechol. △: without Mg^{2+}; ●: with Mg^{2+} (2.5 mM), phosphate buffer (50 mM, pH 7.0), $T = 35°C$.

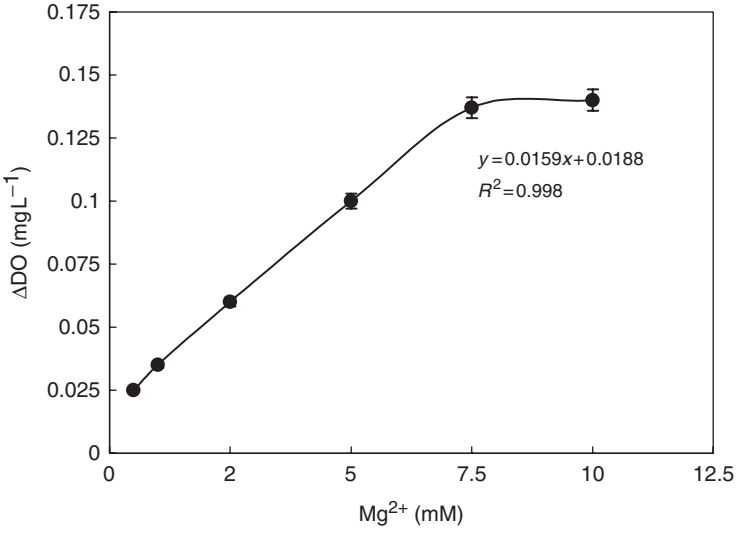

Figure 2. Linear ranges for Mg^{2+}, phosphate buffer (50 mM, pH 7.0), $T = 35^{\circ}C$. Catechol concentration: 2.5 μM.

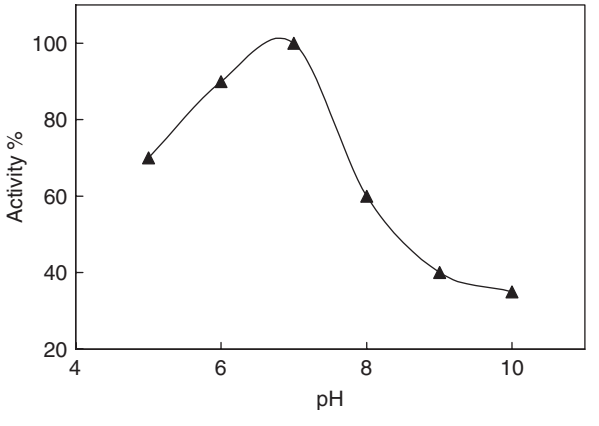

Figure 3. Detection of the effect of pH on the biosensor response (concentration of all buffers is 50 mM, $T = 35^{\circ}C$, Catechol concentration: 2.5 μM and Mg^{2+}: 2.5 mM).

to be 7.0. Below and above this pH value, decreases in the biosensor response were observed.

3.2.2 Effect of temperature on the biosensor response. In order to determine the effect of temperature on the biosensor response, experiments were carried out between 15 and 40°C for catechol (2.5 μM) in the presence of Mg^{2+} ions (2.5 mM). According to the results, the highest biosensor responses were obtained at 35°C. Below and above 35°C, decreases in the biosensor responses were recorded, and figure 4 shows the results obtained.

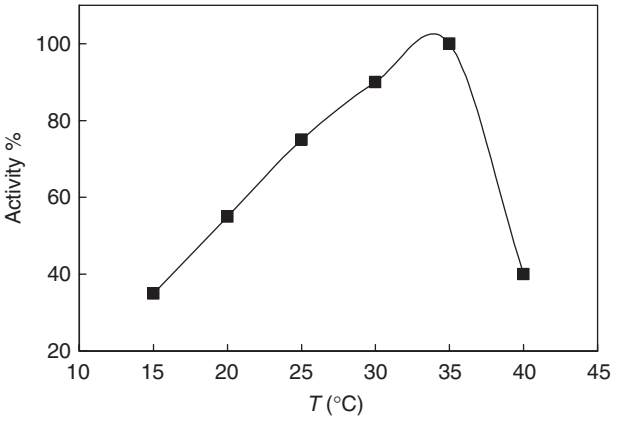

Figure 4. Detection of the effect of temperature on the biosensor response (phosphate buffer 50 mM, pH 7.0; catechol concentration: 2.5 μM and Mg^{2+}: 2.5 mM).

Table 1. Metal activation of the PPO biosensor.[a]

Metal	Activity (%)
Mg^{2+}	100
Cu^{2+}	120
Zn^{2+}	33
Mn^{2+}	20
Ni^{2+}	0

[a]Metal-ion concentration: 1.0 mM.

3.2.3 Investigation of the effects of other metal ions on the biosensor. To investigate the effects of other metal ions on the biosensor response, 1.0 mM of Mg^{2+} ions and the standards of various ions such as Cu^{2+}, Zn^{2+}, Mn^{2+}, and Ni^{2+} were examined in the presence of catechol (2.5 μM). Experiments were carried out in phosphate buffer (50 mM, pH 7.0) and at 35°C. The biosensor response obtained for Mg^{2+} ions was accepted as 100% and compared with the biosensor responses obtained from the other ions (table 1).

3.2.4 Reproducibility. To calculate the reproducibility of the biosensor for Mg^{2+} determination, experiments were also investigated at 2.5 mM Mg^{2+} concentration ($n = 5$) in the presence of 2.5 μM catechol. According to the results obtained, the average value, standard deviation, and variation coefficient (%) were found to be 2.525 mM, 2.50×10^{-2} mM, and 1.0%, respectively.

4. Conclusion

From the results of these experiments, Mg^{2+} ions can be said to play an effective role in the activation of the partially purified PPO enzyme used in the biosensor construction.

Thus, by using the biosensor developed, the concentration of Mg^{2+} ions can be determined in the range of 0.5–7.5 mM by activation of the partially purified PPO in the presence of catechol (2.5 μM) as a substrate. In the optimization of the working conditions of the biosensor, the best results were found to be 35°C and pH 7.0, 50 mM phosphate buffer. When we consider the results of the effects of the other metal ions on the biosensor, it can be said that the biosensor can be used for the determination of both Mg^{2+} and Cu^{2+} ions. Upon comparison with other methods used to determine Mg^{2+} ions, it is obvious that the biosensor can be an alternative method especially for the determination of Mg^{2+} and Cu^{2+} ions.

If we compare the other metal determination methods to the biosensor, it is obvious that the new biosensor method developed is very sensitive, economic, and suitable for routine analysis of Mg^{2+} ions. In addition, by developing and using this type of biosensor, several metal ions and small bio-organic molecules that play a cofactor role in enzymatic reactions can be determined. Thus, it may be said that the biosensor developed may offer a new and alternative strategy for biosensor preparation and technology.

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