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A novel biosensor based on activation effect of thiamine on the activity of pyruvate oxidase

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ABSTRACT

A biosensor based on pyruvate oxidase (POX) enzyme was developed for the investigation of the effect of thiamine (vitamin B_1) molecule on the activity of the enzyme. The biosensor was prepared with a chemical covalent immobilization method on the dissolved oxygen (DO) probe by using gelatin and cross-linking agent, glutaraldehyde. POX catalyzes the degradation of pyruvate to acetylphosphate, CO_2 and H_2O_2 in the presence of phosphate and oxygen. Thiamine is an activator for POX enzyme and determination method of the biosensor was based on this effect of thiamine on the activity of the enzyme. The biosensor responses showed increases in the presence of thiamine. Increases in the biosensor responses were related to thiamine concentration. Thiamine determination is based on the assay of the differences on the biosensor responses on the oxygenmeter in the absence and the presence of thiamine. The biosensor response depend linearly on thiamine concentration between 0.025 and 0.5 μM with 2 min response time. In the optimization studies of the biosensor the most suitable enzyme amount was found as 2.5 U cm^{-2} and also phosphate buffer (pH 7.0; 50 mM) and 35 °C were obtained as the optimum working conditions. In the characterization studies of the biosensor some parameters such as activator and interference effects of some substances on the biosensor response and reproducibility were carried out.

1. Introduction

Thiamine is also known as vitamin B_1 and it helps the body cells to convert carbohydrates into energy. It is also essential for the functioning of the heart, muscles and nervous system. Weakness, fatigue and nerve damage are thiamine deficiency. Vitamin B_1 is very important to the brain, especially in terms of emotional health and well being, and also is useful for focus and concentration. It has an important role in Wernicke–Korsakoff syndrome, a form of amnesia caused by brain damage occurring in long-term alcoholics who rely mainly on alcohol for nutrition. There have been suggestions that vitamin B_1 may have a beneficial effect in treating Alzheimer's disease Wernicke's encephalopathy, peripheral neuropathy, or beriberi heart disease is most often a consequence of chronic alcoholism. Thiamine deficiency is frequently seen in alcoholics because heavy drinking limits the ability of the body to absorb this vitamin from foods. (Gibson et al., 1999; Ba et al., 2005; Pannunzio et al., 2000).

Different types of approaches such as electrochemical including chemosensors and biosensors (Aboul-Kasim, 2000; Akyilmaz et al., 2006; Barrales et al., 1998,2001; Du et al., 2002; Siddiqui and Pitre, 2001; Zhang et al., 1999; Zhu et al., 2003; Zou and Chen, 2007), high performance liquid chromatography (Dinç et al., 2000; Lynch and Young, 2000; Höller et al., 2003; El-Gindy et al., 2004; Bohrer et al., 2004; Losa et al., 2005; Tang et al., 2006), and spectrophotometry (Alonso et al., 2006; Danet and Calatayud, 1994; Liu et al., 2002; Rocha et al., 2003; García et al., 2001) for quantitative determination of thiamine have been reported.

Pyruvate is a key molecule in glycolysis, the tricarboxylic acid cycle and some metabolic processes (Situmorang et al., 2002). Determination of the pyruvate is especially important in the clinical, bioprocess and food analysis. Pyruvate oxidase (POX)-based electrochemical biosensors have been used for this aim (Gajovic et al., 1999; Ghica and Brett, 2006; Akyilmaz and Yorganci, 2007). According to the enzymatic reaction given below pyruvate oxidase uses oxygen and phosphate for catalyzing oxidative decarboxylation of pyruvate to acetylphosphate and hydrogen peroxide.

$$\text{Pyruvate} + \text{Phosphate} + O_2 + H_2O \leftrightarrow \text{Acetylphosphate} + CO_2 + H_2O_2$$

For the catalyzing reaction pyruvate oxidase also required some cofactors such as flavin adenine dinucleotide (FAD), thiamine pyrophosphate (TPP) and magnesium which is needed for catalytic activity of enzyme (Situmorang et al., 2002; Muller et al.,

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1994; Muller and Schulz, 1993; Blake and Hager, 1978). Pyruvate oxidase is a homo-tetrameric enzyme and each subunit (62 kDa) binds to FAD noncovalently and also binds to thiamine diphosphate (TDP) loosely. Cell membrane-associated electron transport system, which includes both ubiquinone-6 and cytochrome *b*, is the natural electron acceptor for the reduced enzyme and oxygen is the terminal electron acceptor of this system (Blake et al., 1982). Mg^{2+} is the metal cation for the reaction which is catalyzed by thiamine pyrophosphate. Pyruvate oxidase has five catalytic steps; deprotonation of C2–H of thiamine diphosphate which is the initial step for all TDP-dependent enzymatic reaction is the first step, in second step enzyme-bound TDP is bound to the C2 atom of pyruvate, third step is decarboxylation of pyruvate to hydroxyethyl-TDP, next step is oxidation of hydroxyethyl-TDP by FAD, and the last step is reoxidation of reduced FAD by oxygen (Tittman et al., 1998).

In this study, in order to benefit from this effect of thiamine a novel biosensor based on the activation effect of thiamine on the pyruvate oxidase enzyme was developed for the thiamine determination.

2. Experimental

2.1. Chemicals

Pyruvate oxidase [Pyruvate:oxygen-2-oxidoreductase(phosphorylating)] (EC 1.2.3.3) (100 U) from *Aerococcus* sp., pyruvic acid sodium salts, thiamine hydrochloride, KH_2PO_4, K_2HPO_4, pyridoxine hydrochloride, nicotinic acid, riboflavin, calf skin gelatin, glutaraldehyde (25%) and all other chemicals were purchased from Sigma Chemical Co. (USA). All solutions were prepared with double distilled water just before their use.

2.2. Apparatus

In this experiments, a YSI Model 58 digital oxygenmeter with 0.01-mg/l dissolved oxygen concentration sensitivity, YSI 5700 model DO probes (with YSI 5740 cable) as transducers, high sensitive teflon membranes (0.0005 in thick) for oxygen (YSI, Yellow Springs, OH, USA), Gilson P100 and P1000 automatic pipets (France), Yellow-Line magnetic stirrer (Germany) and Nuve model thermostat (TR) were used.

2.3. Preparation of the biosensor

First of all DO probe was covered with a high sensitive teflon membrane by using an *O*-ring and then the membrane which is sensitive for oxygen was pretreated with 0.5% sodiumdodecylsulfate (SDS) in phosphate buffer (50 mM, pH 7.0) to reduce the tension on the membrane surface of DO probe. After this step, 210 μl of pyruvate oxidase enzyme solution and gelatin were mixed and dissolved at 38 °C for a few minutes. 200 μl of the solution was spread over the DO probe membrane surface and allowed to dry at 4 °C 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 4 min to form chemical covalent bonds (Schiff bases) between gelatin, enzyme and glutaraldehyde molecules for the immobilization of the enzyme on the surface of the DO probe.

2.4. Measurements

In the reaction, pyruvate oxidase converts pyruvate to acetyl phosphate, carbondioxide and hydrogenperoxide in the presence of oxygen. There is an interval surface between bioactive layer and teflon membrane of DO probe and during the enzymatic reaction dissolved oxygen concentration in the interval surface decreased related to substrate concentration added into reaction medium. The measurements with the biosensor developed were done at steady-state conditions. ΔDO is the difference of dissolved oxygen concentration when the substrate is not in the reaction medium and after addition of substrate into the reaction medium until to obtain a new steady-state DO concentration. It is well known that thiamine is a cofactor for pyruvate oxidase and it plays an activator role for the pyruvate oxidase so when the thiamine was injected into the reaction medium it increased the activity of the enzyme and in this case the dissolved oxygen concentration changed related to the thiamine concentration added into the reaction medium. The principle of the measurement of the biosensor was based on the determination of these changes in dissolved oxygen concentration related to thiamine concentrations used in the enzymatic reaction. As a result, the differences between the first and the last dissolved oxygen concentrations related to thiamine concentrations were detected by the biosensor to obtain a standard curve for the determination of thiamine. All the measurements were done at 35 °C by using a thermostatic reaction cells and the oxygen saturated phosphate buffer (50 mM, pH 7.0).

3. Results and discussion

3.1. Detection of thiamine effect as an activator on the biosensor responses

At the beginning of the study, some experiments were made for the determination of thiamine effect as an activator on the pyruvate oxidase biosensor. For this purpose first, the biosensor developed was used only for pyruvate detection by using standards between (2.5×10^{-3} and 5.0×10^{-2} μM) concentrations in the absence of thiamine and also a linear curve was obtained. After that, by using the same pyruvate standards but in the presence of 0.1 μM thiamine a new standard curve was obtained. Obtained results from the experiments showed that biosensor responses increased very efficiently in the presence of thiamine. Fig. 1 shows this obvious effect on the pyruvate oxidase enzyme activation.

3.2. Optimization of the bioactive surface of the biosensor

3.2.1. Effect of the enzyme activity on the biosensor response

Different enzyme amounts were used for detection the effect of the enzyme activity on the biosensor response. For this purpose three biosensors which contain 1.25, 2.5 and 5.0 U cm^{-2} activity

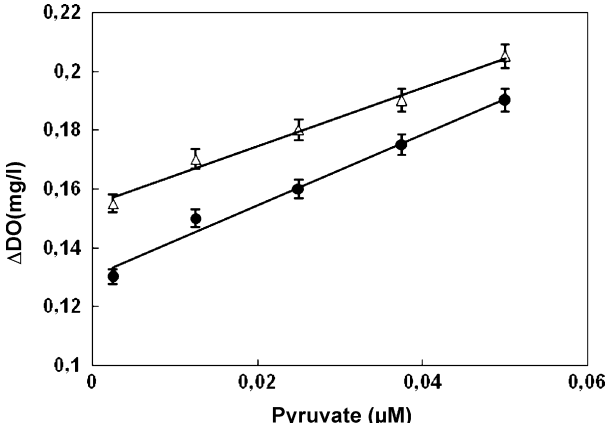

Fig. 1. Determination of the effect of thiamine on the activation of pyruvate oxidase enzyme (phosphate buffer; pH 7.0, 50 mM; *T*: 35 °C; (●) without thiamine (△) with 0.1 μM thiamine). The percentage of glutaraldehyde and gelatine amount were kept constant at 2.5%, and 5.0 mg cm^{-2}, respectively.

of pyruvate oxidase, were prepared by immobilizing with gelatin (5.0 mg cm^{-2}) and glutaraldehyde (2.5%).

According to the results, when the biosensor contained 2.5 or 5.0 U cm^{-2} activity of pyruvate oxidase, the most useful calibration curves were obtained. Thiamine was detected with a linear range between 0.025 and 0.5 μM concentrations by these biosensors. Increase in the pyruvate oxidase activity from 2.5 to 5.0 U cm^{-2} resulted in higher biosensor responses. This was an expected result but the standard curve showed nearly the same responses. When the bioactive layer of the biosensor contained 1.25 U cm^{-2} activity of pyruvate oxidase we did not obtain any suitable standard curve for thiamine. 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 containing 2.5 U cm^{-2} activity of pyruvate oxidase.

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

To detect the effect of the gelatin amounts on the biosensor response different gelatin amounts were used in the construction of the biosensor. Biosensors contained 2.50, 5.0,and 10.0 mg cm^{-2} gelatin, and 2.5 U cm^{-2} pyruvate oxidase. All the biosensors were immobilized by glutaraldehyde (2.5%). The measurements were made in order to obtain standard curves for thiamine. The most suitable curve was obtained with the biosensor prepared by using 5.0 mg cm^{-2} gelatin amount.

When the gelatin amount increases from 5.0 to 10 mg cm^{-2}, we obtained higher biosensor responses but the linearity was swerved. Decreases of the gelatin amounts from 5.0 to 2.5 mg cm^{-2} results in a lower biosensor responses. The reason of this effect was the reduction 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 said that, the most suitable biosensor responses were obtained by the biosensor containing 5.0 mg cm^{-2} gelatin.

3.2.3. 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 2.5 U cm^{-2} pyruvate oxidase and 5.0 mg cm^{-2} gelatin, on the other hand the biosensors were treated with 1.25%, 2.0%, 2.5% and 3.75% glutaraldehyde solution prepared in phosphate buffer (50 mM, pH 7.0) for the immobilization. After immobilization procedure the experiments were made to obtain standard curves for pyruvate by using the biosensors prepared.

From the experiments it can be said that the higher biosensor responses were observed at 1.25% glutaraldehyde percentage. But the linearity was swerved after the 0.1 μM thiamine concentration and after this concentration we detected a second nearly linear range from 0.1 to 0.5 μM. The biosensors, prepared with 2.0% and 3.75% glutaraldehyde, showed lower biosensor responses and the same linearity with a little range. Because of the best linear range and high biosensor responses the biosensors were prepared with 2.5% glutaraldehyde.

3.3. Optimization of working conditions

3.3.1. Effect of pH on the biosensor response

To detect the effect of the pH value on the biosensor response different buffer systems were investigated. For this aim 50 mM concentration of citrate (pH 5.0–6.0), phosphate (pH 7.0–8.0) and glycine (pH 9.0–10.0) buffers were used in the experiments. The optimum pH value was obtained as 7.0. 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 pyruvate oxidase it can be said that the immobilization procedure did not affect the optimum pH value of the enzyme.

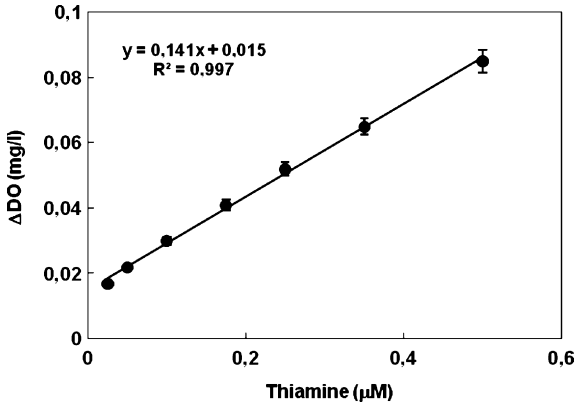

Fig. 2. Standard curve of the biosensor (phosphate buffer; pH 7.0, 50 mM; *T*: 35 °C). The activity of pyruvate oxidase, the concentration of pyruvate, the percentage of glutaraldehyde and amount of gelatin were kept constant at 2.5 U cm^{-2}, 0.025 μM, 2.5%, and 5.0 mg cm^{-2}.

3.3.2. Effect of temperature on the biosensor response

The enzyme activity depends on the temperature and the medium conditions. For the determination of the temperature effect on the biosensor response, the experiments were carried out between 15 and 45 °C. From the experiments, the highest biosensor response was observed at 35 °C, below and above this degree, decreases in the biosensor responses, that probably resulted from the changes in the enzyme structure, were recorded.

3.4. Analytical characteristics of the biosensor

3.4.1. Linear range of the biosensor

The results obtained for the determination of detection limits for thiamine were given in Fig. 2. When we consider the figure, it can be said that the biosensor responses depended linearly on thiamine concentration between 0.025 and 0.5 μM with $y = 0.1419x + 0.0152$ and $R^2 = 0.9979$. The detection limit of the biosensor is 0.025 μM.

3.4.2. Reproducibility

The reproducibility of the biosensor was also investigated for 0.2 μM thiamine concentration ($n = 7$), the average value (x), the standard deviation (S.D.) and coefficient of variation (CV%) were calculated as 0.195 μM, ±0.006 μM, and 3.07%, respectively.

3.4.3. Effect of some compounds on the activity of pyruvate oxidase

In order to detect the effect of different compounds on the pyruvate oxidase activity some experiments were made by using 0.1 μM

Table 1
Effect of some compounds on the activity of pyruvate oxidase

Sample[a]	Biosensor response (mg/l)	Increase in response (%)
Pyruvate	0.11	–
Pyruvate + thiamine	0.13	18.2
Pyruvate + pyrodoxin	0.11	–
Pyruvate + nicotinic acid	0.11	–
Pyruvate + riboflavin	0.11	–

[a] Concentration of the pyruvate is 0.025 μM and the concentration of the other compounds is 0.1 μM.

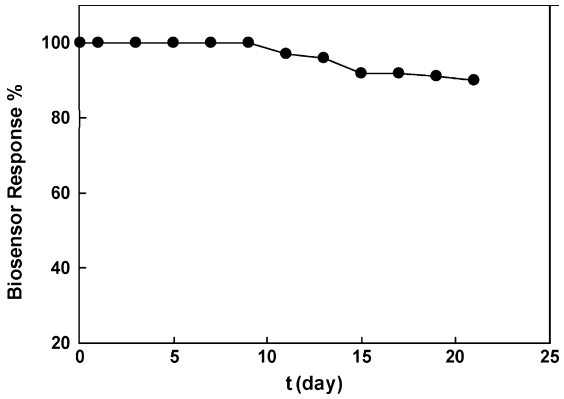

Fig. 3. Long-term stability of the biosensor (phosphate buffer; pH 7.0, 50 mM; *T*: 35 °C). The activity of pyruvate oxidase, the concentration of pyruvate, the percentage of glutaraldehyde and amount of gelatin were kept constant at 2.5 U cm^{-2}, 0.025 μM, 2.5%, and 5.0 mg cm^{-2}. Thiamine concentration is 0.1 μM.

Table 2
Determination of thiamine in vitamin tablets by the biosensor

Sample	Reported (mg/tablet)	Found (mg/tablet)[a]	R.S.D.	CV (%)	Recovery (%)
One a day	1.50	1.56	±0.0132	0.85	104.0
Bemiks	10	10.1	±0.0890	0.88	101.0
Men's one a day	2.25	2.28	±0.0158	0.69	101.3

[a] Average of five measurements.

thiamine and various substances such as pyrodoxin, riboflavin and nicotinic acid at the same concentration with thiamine in the presence of 0.025 μM pyruvate. The increases in the biosensor response obtained with thiamine was compared to other biosensor responses obtained in the presence of these substances (Table 1). According to the results, these substances did not change the biosensor responses and in this case, it can be said that pyrodoxin, riboflavin and nicotinic acid did not play an activator role on the pyruvate oxidase activity.

3.4.4. Long-term stability of the biosensor

One of the most important objectives of enzyme immobilisation on a transducer, for analytical purposes, is to stabilise the enzyme for the biosensor to be used repeatedly over a long period of time. The long-term stability of the biosensor was monitored by measuring its response to 0.1 μM thiamine solution. The biosensor was washed and stored, between measurements, at 4 °C in phosphate buffer solution. From the experiments, the biosensor retained about 10% of its initial activity after 21 days. This result indicates that the immobilisation procedure of the biosensor provides substantial improvement in long-term stability of the biosensor (Fig. 3).

3.5. Application

In this section of the study the thiamine content of some vitamin tablets were determined by using the biosensor. For this purpose, the tablets were pounded and dissolved in double distilled water. After that the solutions were diluted. Results obtained from the experiments were given in Table 2. According to the results it can be said that thiamine in the vitamin tablets can be determined sensitively by using the biosensor.

4. Conclusion

In this study, an amperometric biosensor was developed in order to investigate the effect of thiamine on the activity of pyruvate oxidase enzyme. From the experimental studies we detected a positive effect of thiamine on the enzyme activity and this effect increased in higher thiamine concentrations. By using the biosensor we detected a linear concentration range for thiamine in the presence of constant concentration of pyruvate. Reproducibility of the biosensor is very well and the biosensor can be used as an alternative method for routine analysis of thiamine.

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