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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

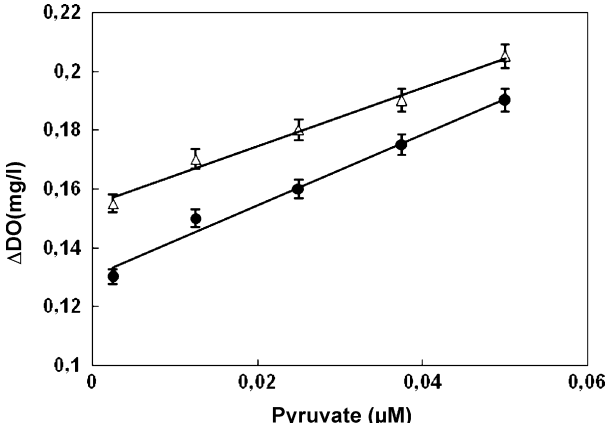

Fig. 1. Determination of the effect of thiamine on the activation of pyruvate oxidase enzyme (phosphate buffer; pH 7.0, 50 mM; *T*: 35 °C; (●) without thiamine (△) with 0.1 μM thiamine). The percentage of glutaraldehyde and gelatine amount were kept constant at 2.5%, and 5.0 mg cm^{-2}, respectively.