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AMPEROMETRIC STUDY OF THE INHIBITORY EFFECT OF BENZOIC ACID ON A TYROSINASE-MODIFIED PLATINUM ELECTRODE

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ABSTRACT. A new type of tyrosinase (PPO)-modified Pt electrode, based on physical entrapment of the enzyme in agar-agar gel, was constructed and used to investigate the inhibitory effect of benzoic acid on the PPO activity. At an applied potential of -50 mV *vs.* SCE, the bioelectrode develops a fast, steady state response, linearly correlated with the phenol concentration up to 0.1 mM, with a sensitivity of 4.2 $mA.M^{-1}.cm^{-2}$. A kinetic interpretation of the amperometric response to phenol recorded in the absence and in the presence of benzoic acid, allowed identifying the inhibition process as a competitive one. The inhibition constant, $(1.16 \pm 0.01)*10^{-6}$ M, and inhibition coefficient, $(1.24 \pm 0.15)\ 10^{-3}$ M, were found in good agreement with similar data published in the literature.

INTRODUCTION

Tyrosinase (polyphenoloxidase, PPO) [1-3] and laccase [4, 5] are enzymes containing binuclear copper, which catalyzes the hydroxylation of monophenols to o-diphenols (cresolase activity) and the oxidation of o-diphenols to o-quinones (catecholase activity). Based on this catalytic scheme, a wide group of bioelectrodes incorporating PPO [6-26] or laccase [27] was built for amperometric detection of phenolic compounds.

On the other hand, it was showed that amperometric biosensors are also very convenient probes for monitoring the inhibition of enzyme activity [28,30]. In this way, these devices extended theirs applications toward detection of some important analytes, which act as enzyme inhibitors, and provided deeper information about the interaction mechanism between substrate and enzyme.

Based on the value of the stability constant (K_I, M^{-1}), corresponding to the inhibitor-enzyme complex, the organic acid inhibitors of the PPO active site can be divided into two main groups (Table 1) [1]. To the first group belong weak inhibitors, bearing a carboxylic function which is not conjugated into the aromatic system ($K_I < 150\ M^{-1}$). The nature of the chain attached to the carboxylic function does not exert a significant effect on the inhibition activity. Contrarily, the second group is composed of strong inhibitors, where the carboxyl is well conjugated into an aromatic ring ($K_I > 1000\ M^{-1}$).

The effect of both organic and inorganic (CN^-, F^-, Cl^-, Br^-, I^-, PO_4^{3-}, CO_3^{2-}, SO_4^{2-}, $B_4O_7^{2-}$, NO_3^-, NO_2^-, N_3^-) inhibitors on the PPO activity are often investigated in literature [28-38]. Generally, this kind of studies was performed with dissolved enzyme in homogeneous systems, using spectrophotometry as monitoring method [39]. However, recently it was proved that the inhibition type can be established and the inhibition parameters can be quantitatively estimated *via* amperometric measurements, examining the biosensor response to its substrate in the presence of the investigated inhibitor [28-38].

Table 1.

The stability constants (K_I) of the complex tyrosinase-inhibitor for some organic acids in aqueous solution [1].

Strong inhibitors acids	**K_I (M^{-1})**	**Weak inhibitors acids**	**K_I (M^{-1})**
m-toluic	450	o-toluic	<10
m-brombenzoic	700	o-brombenzoic	40
benzoic	1400	acetic	10
p-toluic	2000	phenylacetic	110
p-brombenzoic	4100	naphthylacetic	90
p-ethylbenzoic	6800	cyclohexane carboxylic	70
picolinic	570	cycloheptane carboxylic	18
terephthalic	1200		

Starting from the Lineaweaver-Burk equation [40]:

$$\frac{1}{I_{ss}} = \frac{1}{I_m} + \frac{K_m}{I_m}\frac{1}{[S]} \qquad \textbf{(1)}$$

adapted for an amperometric biosensor in the case of a competitive inhibition [39], the reciprocal value of the steady-state response ($1/I_{SS}$) observed for a substrate concentration ([S]) and an inhibitor concentration ([I]) is given by the following equation:

$$\frac{1}{I_{ss}} = \frac{1}{I_m} + \frac{K_m}{I_m}\left(1+\frac{[I]}{K_I}\right)\frac{1}{[S]} \qquad \textbf{(2)}$$

Introducing the inhibition coefficient, $\alpha = (K_m/K_I)$ [31], the equation (2) becomes:

$$\frac{1}{I_{ss}} = \frac{1}{I_m} + \frac{K_m + \alpha[I]}{I_m}\frac{1}{[S]} \quad \textbf{(3)}$$

where: I_m is the intensity of the maxim current and K_m is the apparent Michaelis-Menten constant. Based on equations (1) and (3) a kinetic interpretation of the biosensor responses to substrate, recorded in the absence and in the presence of the inhibitor, allow the estimation of the inhibition parameters α or K_I.

In this work a new amperometric biosensor for phenol detection was developed, by entrapping tyrosinase into agar-agar gel followed by its deposition on a Pt electrode. The aplicability of this kind of bioelectrode for the investigation of enzyme inhibition was checked using benzoic acid, a compound known as a strong inhibitor for PPO [1]. In the same context, in order to check the versatility of the enzyme immobilization technique a similar amperometric biosensor based on laccase was constructed and tested under the same experimental conditions, knowing that the benzoic acid acts for laccase as un uninhibitor [4,5].

EXPERIMENTAL

Reagents

Tyrosinase from mushroom (EC 1.14.18.1; 385 Sigma units/mg) was purchased from Sigma. The laccase extract was prepared as follows. Fresly cutted apple slices and $CaCO_3$ powder, in the ratio 3:1(w/w), were thoroughly mixed for 15 minutes, in a mortar. Then, the necessary amount of distilled water was added in order to obtain a 15 % (w/w) mixture. After 1 hour, the filtered solution can be used as stock solution of laccase.

Electrochemical measurements were made using as supporting electrolyte a 0.1 M $LiClO_4$ solution made in 0.1 M phosphate buffer (pH 7). The phosphate buffer was obtained by mixing the corresponding volumes of 0.1 M KH_2PO_4 and 0.1 M K_2HPO_4.

Phenol, KH_2PO_4, K_2HPO_4 and $LiClO_4$ were obtained from Merck and used as received. The agar-agar powder, $K_4[Fe(CN)_6]$ and $CaCO_3$ were obtained from "Reactivul" Bucharest and were used without any further purification. The apples were purchased from a grocery.

Enzyme electrode preparation

The technique of enzyme entrapment in agar-agar gel [41] consisted in two steps:

(i) 20 mg of agar-agar powder was homogenized with 0.9 mL of 0.1 M $LiClO_4$ in 0.1 M phosphate buffer (pH = 7). The mixture was heated

at 100 ^{o}C and, subsequently, it was cooled at 50 ^{o}C. Then, 1 mL of enzyme solution was added. The concentration of tyrosinase solution was 2.5 mg/mL and it was prepared by dissolving the pure enzyme in distilled water.

(ii) The above-prepared mixture was deposited on a dialysis membrane of 0.3-mm thickness. The so obtained enzyme-modified membrane was stored at 5^{o}C into phosphate buffer (pH 6.5).

In order to investigate the amperometric response of the bioelectrode to substrate (phenol), the enzyme-modified membrane was mechanically attached to a Pt disk electrode (3-mm diameter), taking care to put the enzyme matrix in a close contact with the electrode surface.

Electrochemical measurements

All measurements were performed using a computer-assisted potentiostat (Autolab-PGSTAT-10, Eco Chemie, Utrecht, The Netherlands), connected to a conventional electrochemical cell equipped with three electrodes. The bioelectrode was the working electrode. A saturated calomel electrode (SCE) was used as reference electrode and a Pt-foil as counter electrode in all experiments.

Amperometric measurements were done as follows: the bioelectrode was immersed in 10 ml of testing solution (0.1 M phosphate buffer containing 10^{-6} M $K_4[Fe(CN)_6]$ and 0.1 M $LiClO_4$) at room temperature and poised at the desired value of the applied potential. When the recorded signal attained a stable value, a known volume of standard solution of substrate (phenol) was added under a vigorous stirring. Subsequently, the signal variation corresponding to the reduction of enzymatically produced o-quinone was recorded for 1-2 minutes. Thus, the calibration curve was constructed by mean of successive additions of small volumes of standard aqueous solution of substrate.

Before using the bioelectrode was kept at 5 ^{o}C in a humid atmosphere. The procedure presented above was repeated unchanged in all tests carried out using the amperometric bioelectrode.

RESULTS AND DISCUSSIONS

In order to optimize the behavior of the PPO incorporating bioelectrode, its steady-state amperometric response to phenol was recorded at different enzyme loadings. As it can be seen from figure 1A, an enzyme loading higher than 30 % (w/w) was not productive. Consequently, all further experiments were carried out with bioelectrodes having this enzyme loading.

The temperature dependence of the biosensor response, showed in figure 1B, revealed that the optimum temperature range for enzyme activity was between 20 and 30 ^{0}C. In order to decrease as much as possible the enzyme thermal denaturation for all further investigations, 21 ^{0}C was chosen as working temperature.

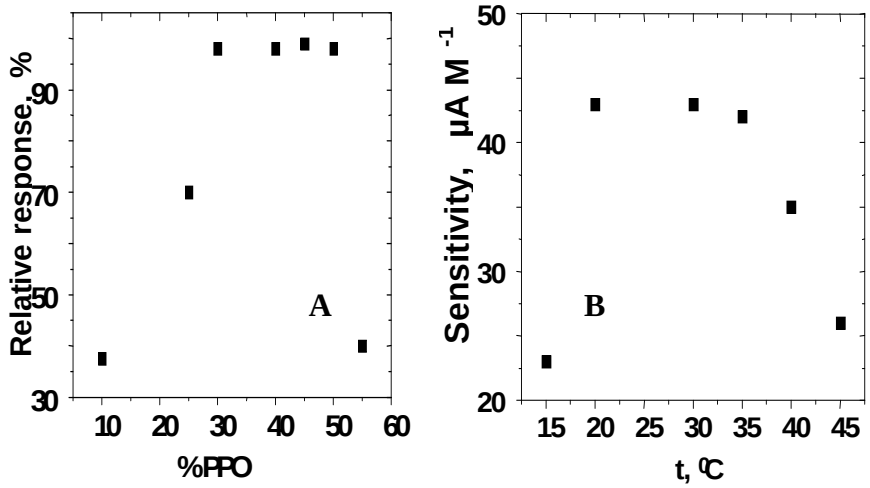


Figure 1. The dependence of the bioelectrode response to phenol on enzyme loading ***(A)*** and on temperature ***(B)***. Experimental conditions: applied potential, -0.18 V *vs.* SCE; phenol concentration, 0.1 mM; supporting electrolyte, 0.1 M phosphate buffer and 0.1 M $LiClO_4$ (pH 7).

As previously reported [6-26], the phenol amperometric detection can be accomplished by applying a potential of -0.2 V *vs.* SCE to detect amperometrically the biocatalytically generated o-quinone. Recently [42], it was proved that using $[Fe(CN)_6]^{4-}$ as mediator it is possible to obtain a significant increase of the bioelectrode selectivity using a lower value of the applied potential (-0.05 V *vs.* SCE), well placed in the optimal domain of the amperometric detection [43]. Operated in this maner, the optimized bioelectrode developed a well-shaped Michaelis-Menten response, proved by the excelent agreement between the experimental data and the calculated curve (Figure 2). The bioelectrode sensitivity to phenol, calculated as the slope of the linear domain, was found (4.2 $mA.M^{-1}.cm^{-2}$) being five times lower than the highest sensitivity reported for PPO containing bioelectrodes [44]. The lower sensitivity and the extended linear domain (up to 0.1 mM) could be attributed to the existance of a diffusion barier, represented by the dialysis membrane used in the bioelectrode construction.

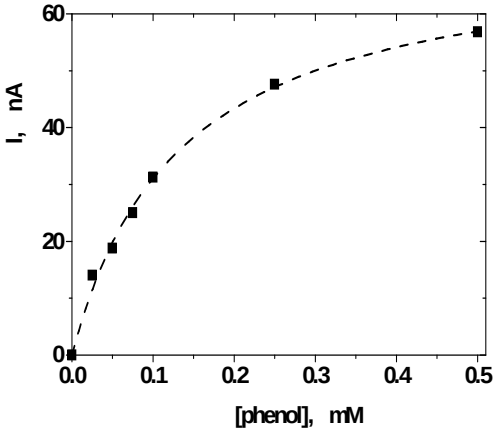
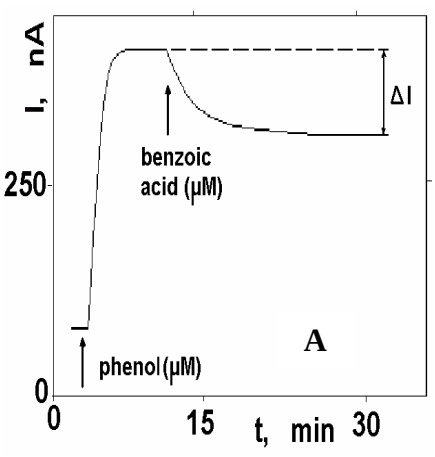


Figure 2. Amperometric response to phenol of the PPO based bioelectrode. Experimental conditions: applied potential, -0.05 V *vs.* SCE; 0.1 M phosphate buffer, pH 7.0, temperature 21^{0}C. The dashed line corresponds to the Michaelis-Menten fitting of the experimental data.

The presence of benzoic acid induced a strong inhibitory effect on the response to phenol of tyrosinase-based bioelectrode (Figure 3A). Contrarily, as it was expected the [4,5], the benzoic acid, in the same domain of concentration, did not exert any influence on the response to phenol for the laccase-based bioelectrode (Figure 3B).



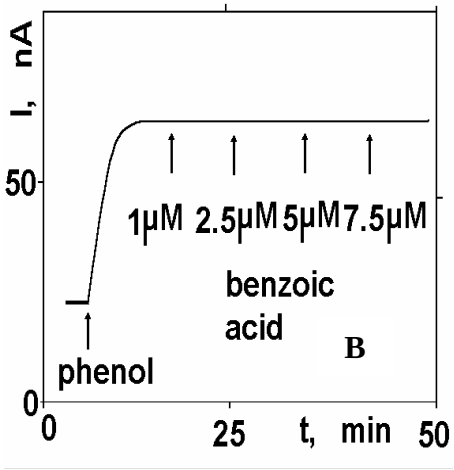


Figure 3A: The amperometric response to phenol of the PPO-based bioelectrode, recorded in the absence and in the presence of 10^{-6} M benzoic acid. Experimental conditions: phenol concentration, 10^{-4} M; applied potential, -0.05 V *vs.* SCE; supporting electrolyte, 0.1 M phosphate buffer and 0.1 M $LiClO_4$ (pH 7); temperature, 21 ^{0}C.

Figure 3B. The amperometric response to phenol of the laccase-based bioelectrode, recorded in the absence and in the presence of the 10^{-6} M benzoic acid. Experimental conditions: as specified for figure 2A.

From the calibration curves to phenol for the PPO-based bioelectrode, recorded in the absence and in the presence of benzoic acid (Figure 4A), and using the equations (2) and (3), in fact from the Linewever-Burk plots (Figure 4B), the characteristic parameters of the inhibition process were estimated (Table 2).

Table 2.

The parameters of the Lineawear-Burk plots for the inhibited and uninhibited response of PPO-based bioelectrode.

Response type	Slope (M/nA)	$1/I_{max}$ (nA^{-1})	Regression coefficient / Number of experimental points
Inhibited	$2.678\ 10^{-6}$	0.0175	0.9965 / 6
Uninhibited	$1.440\ 10^{-6}$	0.0178	0.9908 / 6

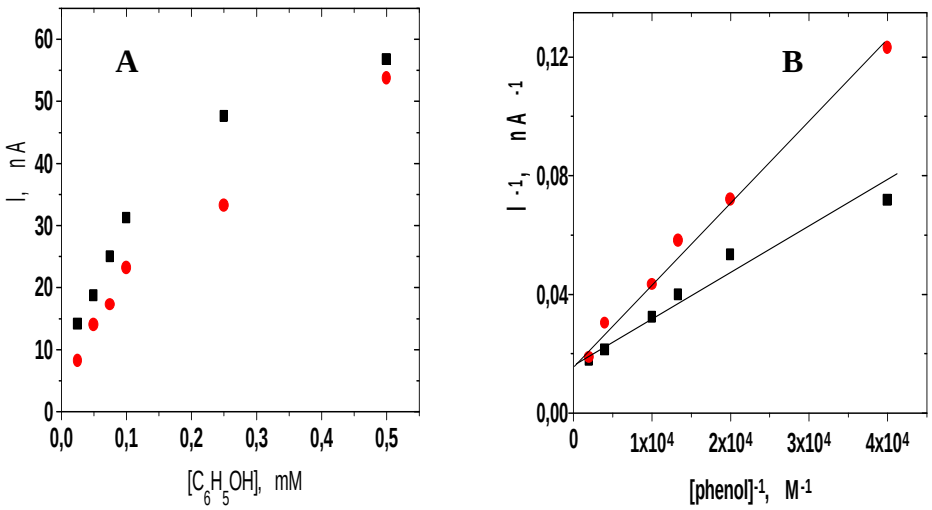


Figure 4. Calibration curves to phenol (**A**) and the corresponding Lineweaver-Burk plots (**B**) for the PPO-based bioelectrode, obtained in the absence (-■-) and in the presence (-●-) of 10^{-6} M benzoic acid. Experimental conditions: applied potential, -0.05 V *vs.* SCE; supporting electrolyte, 0.1 M phosphate buffer and 0.1 M $LiClO_4$ (pH 7); temperature, $21\ ^0$C.

Taking into account that in the both cases (inhibited and uninhibited process) the maximum current intensity had practically the same value (I_{max} ~ 58 nA), it was concluded that the inhibition of the tyrosinase by benzoic acid is competitive with the phenol, at the cresolase active site of the enzyme [39]. The calculated values for the inhibition constant, $(1.16 \pm 0.01)*10^{-6}$ M, and inhibition coefficient, $(1.24 \pm 0.15)\ 10^{-3}$ M, were found in good concordance with that recently published for a PPO-based bioelectrode [45].

CONCLUSIONS

A fast and simple technique for bioelectrode preparation was proposed for the construction of PPO- or laccase- based bioelectrodes. The obtaining procedure of PPO-containing bioelectrode was optimized in respect to the enzyme loading of the enzyme matrix.

A kinetic interpretation of the amperometric response to phenol for the PPO-based bioelectrode, recorded in the absence and in the presence of benzoic acid, allowed identifying the inhibition process as a competitive one. In the same time, no inhibitory effect was noticed for the benzoic acid on the laccase-basaed bioelectrode functioning. In this way it was proved that immobilized in agar-agar gel both enzymes kept their specific bioactivity. Moreover, a comparison with tyrosinase entrapment in amphiphilic polypyrrole [9] showed that the agar-agar gel does not induced a significant loss of enzyme activity.

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