STUDIA UNIVERSITATIS BABEŞ-BOLYAI, CHEMIA, XLIV, 1-2, 1999

BIOSENSORS FOR PHENOL DERIVATIVES USING ELECTROCHEMICAL AND BIOCHEMICAL SIGNAL AMPLIFICATION

SARMIZA ELENA STANCA[1], IONEL CATALIN POPESCU[2], LIVIU ONICIU[2]

[1] *Department of Physics, Chemistry and Informatics, Faculty of Environmental Protection, University of Oradea, 3700 Oradea, Romania*
[2] *Department of Physical Chemistry, University "Babes-Bolyai", 3400 Cluj-Napoca, Romania*

ABSTRACT. Three different approaches to enhance the sensitivity of tyrosinase (PPO) based biosensor for the amperometric detection of phenols have been compared. An electrochemical approach, involving ferrocyanide as mediator for the detection of enzymatically produced o-quinone, was tested using a monoenzyme bioelectrode for phenol. In order to investigate the biochemical approach, based on the cooperative functioning of tyrosinase and D-glucose dehydrogenase (GDH) or tyrosinase and horseradish peroxidase (HRP), two bienzyme electrodes were constructed for L-tyrosine detection at -180 mV *vs.* SCE. For monoenzyme bioelectrode, as well as for bienzyme bioelectrodes, the enzymes were immobized in agar-agar gel. The highest signal amplification factor (74), was observed for the PPO-GDH couple, while that recorded for PPO-HRP couple and PPO-ferrocyanide system were 32 and 4, respectively.

INTRODUCTION

There is a continuous increasing demand for selective and sensitive detection of phenol and its derivatives since these toxic compounds are widely used in the manufacture of various industrial products such as pesticides, disinfectants, fumigants, etc.

Owing to their high selectivity and simple use for continuous on site analysis, biosensors constitute powerful tools for environmental monitoring [1]. In particular, several biosensors based on tyrosinase, a polyphenol oxidase (PPO), were elaborated for the determination of phenol. Since PPO catalyses the oxidation of phenol to o-quinone by dioxygen, various kinds of electrochemical detection were involved in these biosensors: (i) the detection of dioxygen consumption [2-4]; (ii) the direct reduction of the generated o-quinone [5-21]; (iii) the mediated reduction of o-quinone by hexacyanoferrate (II) [22-23], tetracianoquinonedimethane [24], 1,2-naphtoquinone-4-sulphonate [25,26] and N-methylphenazonium [27].

For phenol amperometric biosensors working via the electrochemical reduction of the quinone product a partial substrate recycling was suggested, inducing amplification on the biosensor response [7,11,17-20]. This "intrinsic" amplification effect was supposed to be responsible for the very low detection limits reported for phenol and o-diphenols.

Recently, significant response amplification of PPO-based biosensors has been reported involving: a cyclic chemical reaction between the enzyme-generated o-quinone and a deliberately added reducing agent, as ascorbate [28] or NADH [29]; a cooperative functioning of PPO and horseradish peroxidase (HRP) [30].

In this context, we describe here a study aiming to compare three approaches for response amplification of the tyrosinase (PPO) based biosensor, applied for amperometric detection of phenols. For this purpose mono- and bienzyme electrodes were constructed using agar-agar gel as enzyme immobilization matrix. The electrochemical approach (Figure 1), involving ferrocyanide-mediated reduction of enzymatically produced o-quinone, was tested for phenol detection at PPO monoenzyme bioelectrode. The biochemical approaches, consisting of two enzymes cooperative functioning, have been investigated for PPO and D-glucose dehydrogenase (GDH) couple (Figure 2), as well as for PPO and horseradish peroxidase (HRP) couple (Figure 3). Both bienzyme electrodes were tested for L-tyrosine detection.

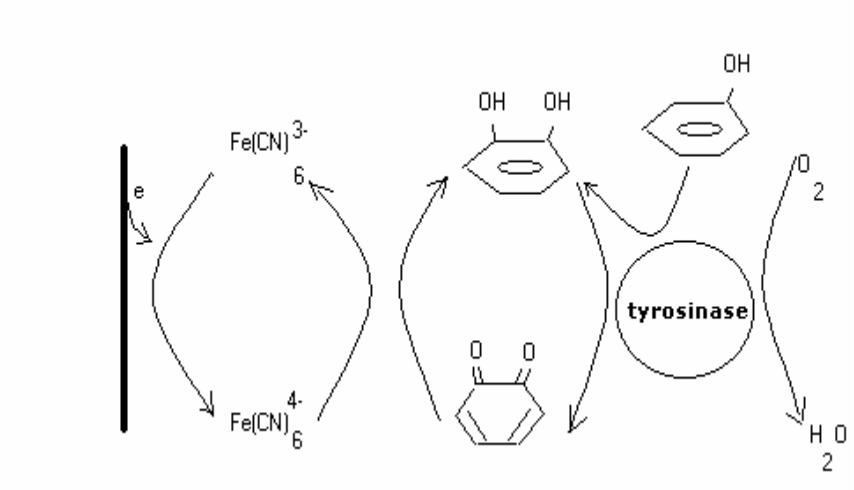

Figure 1. Mediated electro-reduction of o-quinone involved in the signal amplification at PPO-based bioelectrode used for phenol detection [20].

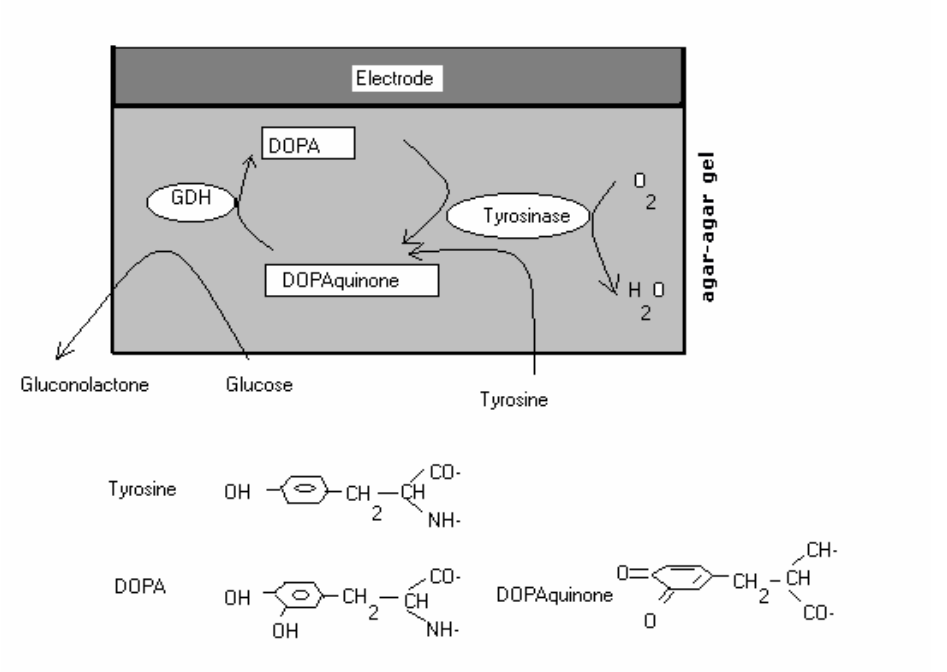

Figure 2. Schematic recycling of DOPA-quinone between tyrosinase and glucose dehydrogenase within a PPO-GDH containing matrix used for L-tyrosine amperometric detection [31].

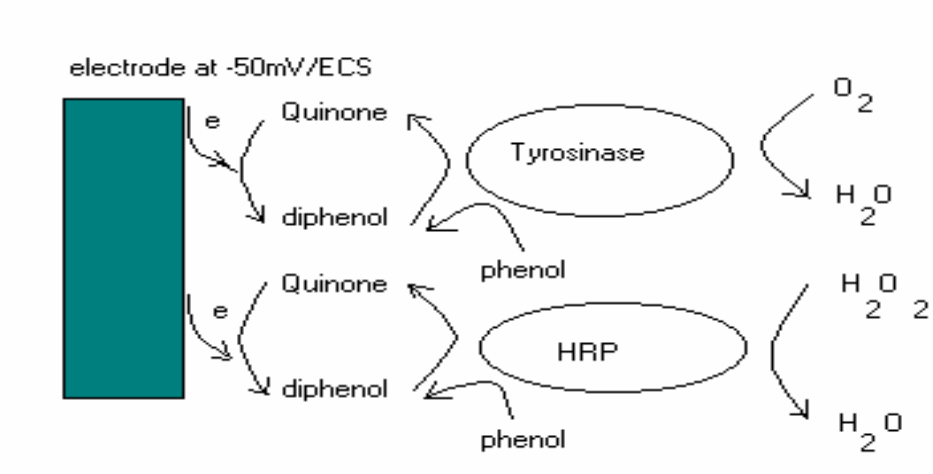

Figure 3. Schematic amplification of the phenol response at PPO-HRP bienzyme bioelectrode [30].

EXPERIMENTAL

Reagents

Tyrosinase from mushroom (EC 1.14.18.1; 385 Sigma units/mg), glucozdehydrogenase (E.C.1.1.1.47; 250 Sigma units/mg) and horseradishperoxidase (EC.1.11.1.7; 250 Sigma units/mg) were purchased from Sigma.

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

Electrochemical measurements were made using as supporting electrolyte 0.1 M $LiClO_4$ in 0.1 M phosphate buffer (pH 6,5 and 7), obtained by mixing the corresponding volumes of 0.1 M KH_2PO_4 and 0.1 M K_2HPO_4.

Enzyme electrode preparation

The technique of enzyme entrapment in agar-agar gel [32] 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). For biosensors using mediated detection, in the above described mixture, 10^{-4} M $K_4[Fe(CN)_6]$ was added. The obtained mixture was heated at 100 ^oC and, subsequently, it was cooled at 50 ^oC. Then, 1 mL of enzyme/enzymes solution was added. The concentration of each enzyme solution was 2.5 mg/mL, and for bienzyme bioelectrodes the enzyme ratio was 1:1(w/w). All enzyme solutions were prepared by dissolving pure enzyme in distilled water.

(ii) the above described mixture was deposited on a dialysis membrane of 0.3 mm thickness. The so obtained enzyme-modified membrane was stored at 5^oC into phosphate buffer at pH = 6.5.

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. In all experiments a saturated calomel electrode (SCE) was used as reference electrode and a Pt-foil as counter electrode.

Amperometric measurements were done as follows: the bioelectrode was immersed in 10 ml of testing solution (0.1 M phosphate buffer containing 0.1 M $LiClO_4$) at room temperature and poised at the desired value of applied potential. When the recorded signal attained a stable value, a known volume of standard solution of substrate (phenol or L-tyrosine) was added, under a vigorous stirring. Subsequently, the bioelectrode amperometric response 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.

RESULTS AND DISCUSSIONS

A. Mediated amperometric detection

A remarkable difference between the response rate corresponding to mediated and unmediated amperometric detection of phenol was noticed for bioelectrodes using tyrosinase entrapped in agar-agar gel (Figure 4). In the same time, as expected, a much greater sensitivity (400 μA/M) for mediated detection than for unmediated process (98 μA/M) was estimated, as the slope of the linear domain, from the calibration curve shown in figure 5.

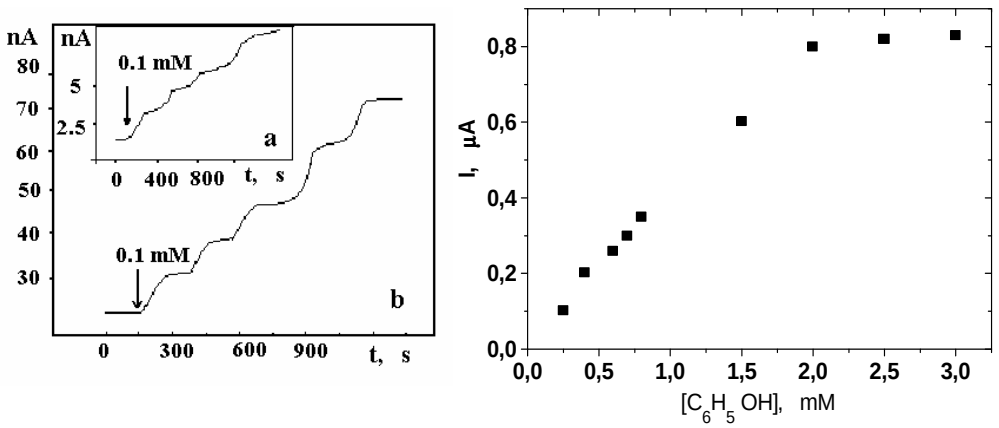

Figure 4. Amperometric response to phenol recorded for the bioelectrode based on tyrosinase entrapped in agar-agar gel: without ***(a)*** and in the presence of $[Fe(CN)_6]^{4-}$ ***(b)***. Experimental conditions: applied potential, -180 mV *vs.* SCE; pH 7, stirred solution; t 21 ^{0}C.

Figure 5. Calibration curve to phenol for tyrosinase-based biolectrode using mediated amperometric detection. Experimental conditions: as indicated for figure 4.

The rate constant (k_{med}) corresponding to the reaction between the redox mediator and the biochemically-produced o-quinone is an important parameter characterizing the intensity of the electrocatalytic effect. A very efficient method for k_{med} evaluation is based on the value of the catalytic limiting current (I_k), described by equation 1 [33]:

$$I_k = nFAC_{med}\sqrt{2D_{med}k_{med}[E]} \quad (1)$$

where: n, represents the number of transfered electrons; A, stands for the electrode surface; C_{med} is the bulk concentration of the mediator; [E] stands for

the total enzyme concentration; D_{med} is the diffusion coefficient of the mediator in solution. Taking n = 1; [E] = 2.5 mg/mL; A = 7.07 mm^2; C_{med} = 4.5 μM; D_{med} = $7.8 \cdot 10^{-6}$ cm^2/s, the k_{med} value was estimated at (1.3 ± 0.04) 10^5 $M^{-1}s^{-1}$, which was found in good agreement with published data [33].

B. Bienzyme cooperative functioning

A comparison of the amperometric responses to L-tyrosine was done between monoenzyme PPO-based bioelectrode and bienzyme bioelectrodes using PPO-GDH and PPO-HRP enzyme couples. The obtained results are presented in figure 7.

In all cases, in order to facilitate the comparison, the enzyme matrix contained the same amount of PPO, and the amperometric detection was performed measuring the current intensity corresponding to o-quinone reduction.

As it can be seen, the highest response was recorded for PPO-GDH couple and the lowest for the PPO-based bioelectrode, using as detection process the mediated o-quinone electro-reduction. This sequence of bioelectrochemical responses is in good agreement with recently published results about the beneficial effect of H_2O_2-HRP couple on the phenol-PPO reaction [30], and about the efficient substrate recycling evidenced for PPO-GDH couple [31].

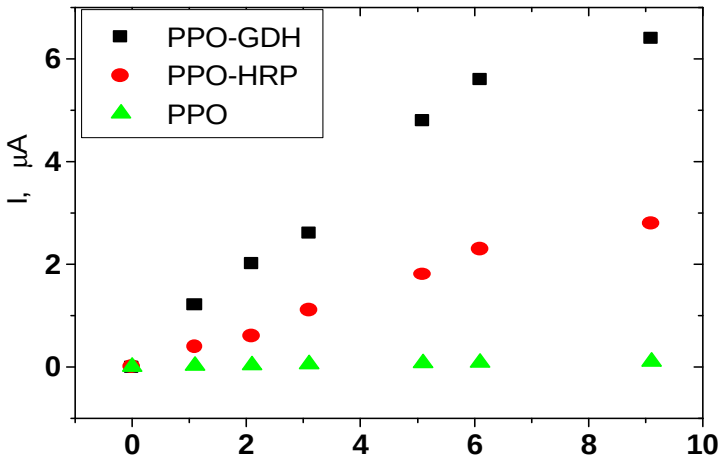

Figure 7. Calibration curves to L-tyrosine for monoenzyme (PPO) bioelectrode and bienzyme bioelectrodes (PPO-GDH and PPO-HRP). Experimental conditions: applied potential, -180 mV *vs.* SCE; supporting electrolyte, 0.1 M fosfat buffer containing $LiClO_4$ 0.1M (pH 6.5); stirred solution; 21 ^{0}C; for PPO-HRP bioelectrode, 1 mM H_2O_2 was added; for PPO-GDH bioelectrode, 2 mM glucose was added.

The bioelectrochemical parameters, I_{max} and K_m, for the three investigated bioelectrodes were estimated using the Lineweaver-Burk linearization of the bioelectrodes response to L-tyrosine, and together with the bioelectrodes sensitivity are presented in table 1.

Table 1.

The bioelectrocemical parameters corresponding to L-tyrosine response of PPO, PPO-GDH and PPO-HRP based bioelectrodes.

Enzyme matrix	Sensitivity (mA/M)	I_{max} (μA)	K_m (mM)
PPO	**0.010**	**0.196**	**10**
PPO-HRP	**0.326**	**19.6**	**55**
PPO-GDH	**0.741**	**16.4**	**14**

It is interesting to remark that comparing the performances of PPO and PPO-GDH bioelectrodes, despite the dramatic sensitivity increase (74 times), the K_m value remained practically unchanged. This behavior confirms the response amplification scheme presented in figure 2. Contrarily, the PPO-HRP bioelectrode, besides an improved sensitivity (32 times) showed a significant higher value for K_m than the specific value for PPO. This K_m increase suggests a decrease of the substrate-enzyme affinity or a supplementary diffusion constraint exisiting in the enzyme matrix, both associated with the increase of enzyme activity, induced by the presence of H_2O_2 [31].

CONCLUSIONS

The mediated scheme for o-quinone detection allowed sensitivity increasing of 4 times compared with a similar biosensor using the direct detection. The phenol biosensor based on ferocianide recycling was found very convenient for aqueous solution using. Simple construction, robustness and a relative long lifetime (more than 3 months) characterized it.

Taking into account the sensitivity and the bioelectrochemical parameters of the investigated bioelectrodes, the biochemical approach for signal amplification based on GDH-PPO couple was found the most suitable for L-tyrosine detection. Moreover, the PPO-GDH bioelectrode showed, besides the highest sensitivity, the shorter response time.

When entrapped in the agar-agar gel all enzymes kept their specific activity, pointing out this matrix as a very convenient one for enzyme immobilization.

ACKNOWLEDGEMENT

The Institute for Instrumental Analysis, Research Center, Karlsruhe (Germany), the Agronomy Faculty from Cluj Napoca (Romania) and the Medicine Faculty from Oradea (Romania) are gratefully acknowledged for their generous supply of enzymes, L-tyrosine and dialysis membrane, respectively.

REFERENCES

1. D. Griffits and G. Hall, TIBTECH, 1993, **11**,122.
2. L. Macholan and L. Schanel, Collect. Czech. Chem. Commun., 1977, **42**, 3667.
3. L. Campanella, M.P. Sammartino, and M. Tomassetti, Sens. Act. **B**, 1992, **7**, 383.
4. S. Canofeni, S. Di Sario, J. Mela and R. Pilloton, Anal. Lett., 1994, **27**, 1659.
5. G.F. Hall, D.J. Best and A.P.F. Turner, Anal. Chim. Acta, 1988, **213**, 113.
6. P. Skladal, Collect. Czech. Chem. Commun., 1991, **56**, 1427.
7. S. Cosnier and C. Innocent, J. Electroanal. Chem., 1992, **328**, 361.
8. J. Wang, N. Naser, H.-S. Kwon and M.Y. Cho, Anal. Chim. Acta, 1992, **264** 7.
9. J. Wang, A.J. Reviejo and S. Mannino, Anal Lett., 1992, **25**, 1399.
10. F. Ortega, E. Dominguez, G. Jönsson-Pettersson and L. Gorton, J. Biotechnol., 1993, **31**, 289.
11. S. Cosnier and C. Innocent, Bioelectrochem. Bioenerg., 1993, **31**, 147.
12. J. Wang, Y. Lin and Q. Chen, Electroanalysis, 1993, **5**, 23.
13. J. Wang, Y. Lin and L. Chen, Analyst, 1993, **118**, 277.
14. J. Wang, L. Fang and D. Lopez, Analyst, 1994, **119**, 455.
15. J.L. Besombes, S. Cosnier, P. Labbé and G. Reverdy, Anal Lett., 1995, **28**, 405.
16. L. Coche-Guérente, S. Cosnier and C. Innocent, Anal. Lett., in press.
17. J. Wang, F. Lu and D. Lopez, Biosens. Bioelectron., 1994, **9**, 9.
18. F. Liu, A. J. Reviejo, J.M. Pingarrón, Talanta, 1994, **41**, 455-459.
19. M. Lutz, E. Burestedt, J. Emnéus, H. Lidén, S. Gobhadi, L. Gorton and G. Marko-Varga, Anal. Chim. Acta, 1995, **305** 8.
20. P. Önnerfjord, J. Emnéus, G. Marko-Varga, L. Gorton, F. Ortega and E. Domínguez, Biosens. Bioelectron., 1995, **10**, 607.
21. C. Nistor, J. Emneus, L. Gorton, L. and A. Ciucu, Anal. Chim. Acta, 1999, **387**, 309.
22. M. Bonakdar, J.L. Vilchez and H.A. Mottola, J. Electroanal. Chem., 1989, **226**, 47.
23. P.I. Ortiz, P.R.A. Nader and H.A. Mottola, Electroanalysis, 1993, **5**, 165.
24. J. Kulys and R. D. Schmid, Anal. Lett., 1990, **23**, 589.
25. M.H. Smit and G.A. Rechnitz, Electroanalysis, 1993, **5**, 747.
26. W.R. Everett and G.A. Rechnitz, Anal.Chem., 1998, **70**, 807.
27. H. Kotte, B. Grundig, K. D. Vorlop, B.Stuhlitz, U. Stottmeister, Anal. Chem., 1995, **67**, 3922.
28. S. Uchiyama, Y. Hasebe, H. Shimizu and H. Ishihara, Anal. Chim. Acta, 1993, **276**, 341.
29. R. S. Brown, K.B. Male and J.H.T. Luong, Anal. Biochem., 1994, **222**, 131.

30. S. Cosnier and I.C. Popescu, Anal. Chim. Acta, 1996, 3**19**, 145.
31. A.V. Eremenko, A. Makower, C.G. Bauer, I.N. Kurochkin, F.W. Scheller, Electroanalysis, 1997, **9**, 288.
32. A.E. Cass, *Biosensors. A practical Approach*, IRL Oxford University Press, 1990, p. 2-262.
33. G. Robinson, D. Leech and M.R. Smyth, Electroanalysis, 1995, **7**, 952.