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Amperometric study of the inhibitory effect of carboxylic acids on tyrosinase

Sarmiza Elena Stanca [a,*], Ionel Catalin Popescu [b]

[a] *Laboratory for Photonics and Interfaces, Institute of Molecular and Biological Chemistry, Swiss Federal Institute of Technology, Lausanne, Switzerland*
[b] *Department of Physical Chemistry, University "Babes-Bolyai", Cluj-Napoca 3400, Romania*

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Abstract

A tyrosinase-modified Pt electrode, based on physical entrapment of the enzyme in agar–agar gel, was constructed and used to investigate the inhibitory effect of six carboxylic acids. At an applied potential of −50 mV versus saturated calomel electrode (SCE), the bioelectrode develops a fast, steady state response, linearly correlated with the phenol concentration up to 10 mg/l, with a sensitivity of 3.7 nA l/mg. A kinetic analysis of the amperometric response to phenol, recorded in the absence and in the presence of carboxylic acids (benzoic, 3-bromobenzoic, 4-ethylbenzoic, acetic, phenylacetic, 2-naphthylacetic acids), revealed that for the first four compounds the inhibition process corresponds to an uncompetitive one. Using the Lineweaver–Burk linearization the inhibition constants as well as the inhibition coefficients were calculated for the strong inhibitors: benzoic, 3-bromobenzoic, 4-ethylbenzoic and acetic acids.

Keywords: Tyrosinase; PPO inhibitors; Agar–agar gel

1. Introduction

The development of amperometric biosensors continues to be a rapidly growing research field. Recently, it was shown that a wide group of phenols could be detected employing amperometric biosensors incorporating tyrosinase (polyphenoloxidase, PPO), in aqueous [1–9] as well as in non-aqueous media [10].

PPO is an enzyme containing binuclear copper, which catalyses the hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the oxidation of *o*-diphenols to *o*-quinones (catecholase activity) [11–13]. Using the reduction either of oxygen or quinone, species present during phenol oxidation [11], several bioelectrodes incorporating PPO were proposed for phenols detection [14–34]. On the other hand, it was shown that amperometric biosensors are very convenient probes for monitoring the enzyme inhibition, too [35–42]. Consequently, taking into account the intrinsic advantages of amperometric biosensors such as easy production, low cost, simple handling and good analytical characteristics (high sensitivity and response rate, extended linear range and low detection limit), amperometric biosensors are recommended as promising sensors in warning devices for accidental water pollution [36].

The effect of organic inhibitors, such as benzoic acid and its derivatives [35,41], kojic acid and its derivatives [40,41], hydrazine [38], thiourea and its derivatives [39,41], methyl and propyl 4-hydroxybenzoate [41], as well as the effect of inorganic inhibitors, such as CN^-, F^-, Cl^-, Br^-, I^-, PO_4^{3-}, CO_3^{2-}, SO_4^{2-}, $B_4O_7^{2-}$, NO_3^-, NO_2^-, N_3^-; [11,41,42], on the PPO activity was noticed and carefully investigated. Generally, this kind of study was performed on dissolved enzyme, using spectrophotometry as monitoring method [43]. Nevertheless, recently it was proven that the inhibition mechanism can be investigated and the inhibition parameters can be quantitatively estimated by using amperometric measurements, simply examining the biosensor response to the enzyme substrate in the presence of the investigated inhibitor [35–42]. The amperometric procedure eliminates the experimental inconveniences (sample pre-treatment, high qualified personnel, and sophisticated instrumentation) related to

* Corresponding author. Present address: Department of Physics, Chemistry and Informatics, Faculty of Environmental Protection, University of Oradea, Oradea 3700, Romania.
Tel.: +40-59-42550/+41-21-693-3669; fax: +41-21-693-4111.
E-mail addresses: stanca@rdslink.ro, sarmizaelena.stanca@epfl.ch (S.E. Stanca).

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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 (l/mg)	Weak inhibitors acids	K_I (l/mg)
3-Methyl benzoic	61.272	2-Methyl benzoic	<1.36
3-Bromobenzoic	140.000	2-Bromobenzoic	8.040
Benzoic	170.800	Acetic	0.600
4-Methyl benzoic	272.320	Phenylacetic	15.070
4-Bromobenzoic	824.100	Naphthylacetic	18.210
4-Ethylbenzoic	1020.000	Cyclohexane carboxylic	8.260
2-Pyridinecarboxilic	129.960	Cycloheptane carboxylic	2.376
1,4-Benzenedicarboxylic	25.579		

the spectrophotometry [43]. At the same time, the amperometric method is preferred due to the short time of analysis, and especially due to its possibility to perform "in situ" measurements.

Using the Lineweaver–Burk equation adapted for the response of an amperometric biosensor in the case of a competitive inhibition [44,45]:

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

where: $(1/I_{ss})$, stands for the reciprocal value of the steady-state response developed for $[S]$ substrate concentration and $[I]$ inhibitor concentration; I_m, the maximum current intensity; K_m, the apparent Michaelis–Menten constant; $\alpha = (K_m/K_I)$, is the inhibition coefficient [44] a kinetic interpretation of the biosensor response to substrate, recorded in the absence and in the presence of the inhibitor, allows the estimation of the inhibition parameters α and K_I.

In this work, by entrapping tyrosinase into agar–agar gel followed by its deposition on a Pt electrode, an amperometric biosensor for phenol detection was developed. The applicability of this bioelectrode for investigation of PPO inhibition was checked using benzoic, *p*-bromobenzoic and *p*-ethylbenzoic acids, compounds known as strong inhibitors for PPO (Table 1), as well as acetic, phenylacetic, naphthylacetic acids known as weak inhibitors (Table 1). The inhibition constants and the inhibition coefficients corresponding to 3-brombenzoic, 4-ethylbenzoic and acetic acids were for the first time reported.

2. Experimental

2.1. Reagents

Tyrosinase from mushroom (EC No. 1.14.18.1; 385 Sigma U/mg) was purchased from Sigma. Benzoic (EC No. 2006182), 3-brombenzoic (EC No. 2095623), 4-ethylbenzoic (EC No. 2106053), acetic (EC No. 2005807), phenylacetic (EC No. 2031486), 2-naphthylacetic (EC No. 2094750) acids and phenol (EC No. 2036327), KH_2PO_4 (EC No. 2319134) K_2HPO_4 (EC No. 2318345) and $LiClO_4$ (EC No. 2322372) were obtained from Fluka and used as received.

The $K_4[Fe(CN)_6]$, 1,2-dihydroxybenzene, 1,3-dihydroxybenzene, 1,4-dihydroxybenzene, 2-amino-3(4-hydroxyphenyl)propanoic, 2-hydroxytoluene, 3-hydroxytoluene, 4-hydroxytoluene, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 4-hydroxybenzoic acid and $CaCO_3$ were obtained from "Reactivul" (Bucharest, Romania) and were used without any further purification.

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

2.2. Enzyme electrode preparation

The protocol of enzyme entrapment in agar–agar gel [46] consisted in two steps:

(i) 20 mg of agar–agar powder was homogenised with 0.9 ml of 0.1 M $LiClO_4$ in 0.1 M phosphate buffer pH 7. The mixture was heated to 100 °C and subsequently cooled to 50 °C. Then, 1 ml of enzyme solution (2.5 mg PPO/ml of distilled water) was added.
(ii) The above-prepared mixture was deposited on a dialysis membrane of 0.3 mm thickness. The enzyme-modified membrane was stored at 5 °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.

2.3. Electrochemical measurements

All measurements were performed using a computer-assisted potentiostat (Autolab-PGSTAT-10, EcoChemie, Utrecht, The Netherlands), connected to a conventional three electrodes electrochemical cell. 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 of pH 7, containing 1 mM $K_4[Fe(CN)_6]$ and 0.1 M $LiClO_4$) at room temperature and maintained at the desired value of the applied potential. When the recorded current signal attained a stable value, a known volume of standard solution of substrate (phenol) was added under vigorous stirring. Subsequently, the signal variation corresponding to the reduction of enzymatically-produced *o*-quinone was recorded for 1–2 min. Thus, a calibration curve was constructed by

means of successive additions of small volumes of substrate standard solution.

Before use the bioelectrode was kept at 5 °C in a humid atmosphere. The procedure presented above was repeated unchanged in all tests carried out with the amperometric bioelectrode.

3. Results and discussions

In our previous study dedicated to the obtaining of a PPO-agar–agar gel biosensor [47], its steady-state amperometric response to phenol was recorded at different enzyme and agar–agar loadings. An enzyme loading higher than 50% (w/w) was proven to be not productive. Consequently, all further experiments were carried out with bioelectrodes having this enzyme loading.

The temperature dependence of the biosensor response revealed that the optimum temperature range for enzyme activity was between 20 and 25 °C [47]. For decreasing as much as possible the enzyme thermal denaturation for all further investigations, 21 °C was chosen as working temperature.

Regarding the pH influence on the PPO-based biosensor response the optimal pH range was found between 5.5 and 7.5. Outside of this interval a significant decrease, almost linear with pH variation, was observed [47].

The selectivity of the phenol biosensor toward different phenolic compounds was checked using their aqueous solutions (Table 2). A comparison with tyrosinase entrapped in an amphiphilic polypyrrole matrix [16] showed that the agar–agar gel does not induced a significant loss of enzyme activity and allow a fast and non-expensive kinetic study.

Usually, the phenol amperometric detection is performed by applying a potential of −0.2 V versus SCE in order to detect the biocatalytically-generated *o*-quinone [13]. Recently [48], it was proven that using $[Fe(CN)_6]^4$ as mediator for *o*-quinone detection it is possible to increase the bioelectrode selectivity by using a less negative applied potential (−0.05 V versus SCE), well placed in the optimal domain of the amperometric detection [49]. In these conditions, the bioelectrode sensitivity to phenol (calculated as the slope of the linear domain) was found of 3.7 nA l/mg, and a linear domain up to 10 mg/l was noticed.

Table 2
The selectivity of the Pt/agar–agar-PPO biosensor

Phenolic compounds	Relative signal (%)
Phenol	100
1,2-Dihydroxybenzene	200
1,3-Dihydroxybenzene	0
1,4-Dihydroxybenzene	1
2-Amino-3(4-hydroxyphenyl)propanoic	50
2-Hydroxytoluene	1
3-Hydroxytoluene	125
4-Hydroxytoluene	160
4-Chlorophenol	20
3-Chlorophenol	12
2-Chlorophenol	1
4-Hydroxybenzoic acid	2.2

Experimental conditions: applied potential, −0.05 V vs. SCE; phenol concentration 0.11 mg/l, the other phenolics concentrations correspond for the same molarity; supporting electrolyte, 0.1 M $LiClO_4$ in 0.1 M phosphate buffer (pH 7); temperature, 21 °C. The current intensity of 23 μA corresponds to 100%.

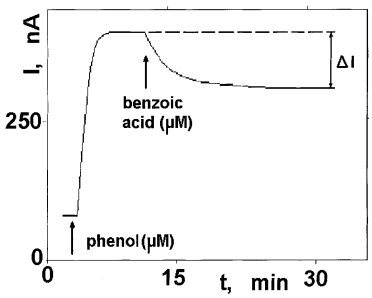

Fig. 1. The amperometric response to 0.11 mg/l phenol recorded for the PPO-based bioelectrode, in the absence and in the presence of 10^{-6} M benzoic acid. Experimental conditions: phenol concentration, 10^{-4} M; applied potential, −0.05 V vs. SCE; supporting electrolyte, 0.1 M $LiClO_4$ in 0.1 M phosphate buffer (pH 7); temperature, 21 °C.

The presence of benzoic acid induced a strong inhibitory effect on the response to phenol of the PPO-based bioelectrode (Fig. 1). The calibration curves to phenol for the PPO-based bioelectrode, recorded in the absence and in the presence of the investigated inhibitors (Fig. 2A), were

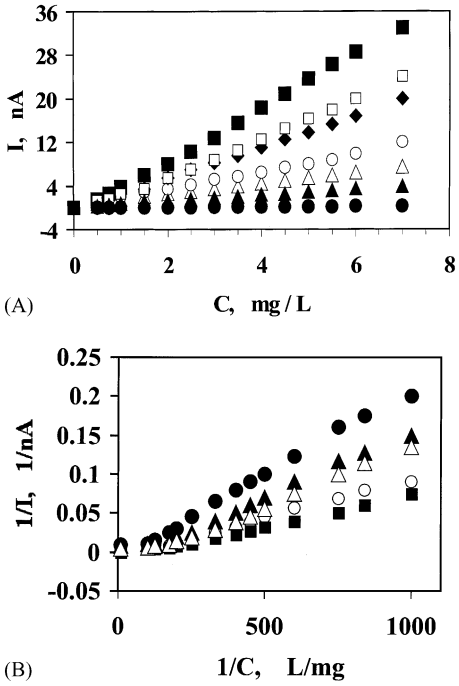

Fig. 2. (A) Calibration curves to phenol and (B) the corresponding Lineweaver–Burk plots, for the PPO-based bioelectrode, obtained in the (■), absence and (△), in the presence of 122 μg/l benzoic; (▲), 3-bromobenzoic; (●), 4-ethylbenzoic; (○), acetic; (□), phenylacetic and (◆), 2-naphthylacetic acids. Experimental conditions: applied potential, −0.05 V vs. SCE; supporting electrolyte, 0.1 M phosphate buffer (pH 7); temperature, 21 °C.

Table 3
The parameters of the Lineweaver-Burk plots for the inhibited and uninhibited response of the PPO-based bioelectrode

Biosensor response	Inhibitor	Slope (nA l/mg)	$1/I_{max}$ (nA^{-1})	Correlation coefficient/number of experimental points
Inhibited	Benzoic acid	3.9 ± 0.1	0.0175 ± 0.0002	0.9965/14
Inhibited	3-Brombenzoic acid	3.2 ± 0.1	0.0178 ± 0.0001	0.9874/14
Inhibited	4-Ethylbenzoic acid	2.21 ± 0.09	0.0178 ± 0.0001	0.9968/14
Inhibited	Acetic acid	6.25 ± 0.07	0.0171 ± 0.0002	0.9923/14
Uninhibited		15.3 ± 0.3	0.0178 ± 0.0003	0.9908/14

interpreted using the Lineweaver–Burk plots (Fig. 2B). Finally, the parameters describing the inhibition process were estimated using the I_m values and Lineweaver–Burk plots slopes (Table 3), and the Eq. (1).

Taking into account that the maximum current intensity has practically the same value ($I_m \sim 58$ nA), irrespective of inhibitors presence or absence, it was concluded that benzoic, 3-brombenzoic, 4-ethylbenzoic and acetic acids exert a competitive inhibition, located at PPO cresolase active site [47]. Contrarily, in similar experimental conditions, any significant inhibition was observed for phenylacetic and 2-naphthylacetic acids.The calculated values for the benzoic acid inhibition constant ($K_I = 70.11 \pm 0.01$ l/mg) and its inhibition coefficient ($\alpha = 7.09 \pm 0.05$ l/mg) were found in good agreement with those published for a PPO-containing bioelectrode, based on oxygen consumption measurement [35,41]. The "apparent" kinetic data for 3-brombenzoic acid, 4-ethylbenzoic acid and acetic acid, such as the inhibition constants (100.11 ± 0.01) l/mg, (140.11 ± 0.01) l/mg, (8 ± 0.01) l/mg and the inhibition coefficients, (11.23 ± 0.03) l/mg, (14.3 ± 0.02) l/mg, (0.81 ± 0.02) l/mg have been estimated, too.

A clear correlation between the inhibitor acidity and their inhibition activity was noticed. Thus, the sequence of the decreasing inhibition constants:

$$4 - \text{ethylbenzoic acid} > 3 - \text{bromobenzoic acid} > \text{benzoic acid} > \text{acetic acid}$$

corresponds to the sequence of the increasing inhibitors acidity. A possible explanation for this similarity could be offered by the fact that oxy-, met- or desoxy-PPO active centres have a strong affinity for HO groups and, consequently, their acid dissociation will weaken this interaction, diminishing the inhibitory effect of the stronger acids.

4. Conclusions

We have developed a fast, simple and reliable technique for monitoring the inhibitory effect of some carboxylic acids on PPO via its incorporation in an amperometric biosensor. A PPO-modified Pt electrode, based on physical entrapment of the enzyme in agar–agar gel, gave a fast, steady state response, linearly correlated with the phenol concentration up to 10 mg/l, with a sensitivity of 3.7 nA l/mg.

The kinetic interpretation of the amperometric response to phenol for the PPO-based bioelectrode, recorded in the absence and in the presence of benzoic, 3-bromobenzoic, 4-ethylbenzoic, acetic, phenylacetic and 2-naphthylacetic acids, allowed identification of an inhibition process of a competitive type, in the case of first four acids. Among the investigated inhibitors, 4-ethylbenzoic exhibits the greatest inhibition effect being followed by 3-bromobenzoic acid, benzoic acid and acetic acid. Using the "apparent" kinetic data a correlation between the inhibitors acidity and their inhibition effect was revealed.

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References

[1] B. Eggins (Ed.), Biosensors An Introduction, Wiley, Weinheim, New York, 1996, p. 215.
[2] S. Cosnier, C. Innocent, J. Electroanal. Chem. 328 (1992) 361.
[3] S. Cosnier, C. Innocent, Bioelectrochem. Bioenerg. 31 (1993) 147.
[4] J. Wang, L. Fang, D. Lopez, Biosens. Bioelectron. 9 (1994) 9.
[5] F. Liu, A.J. Reviejo, J.M. Pingarron, J. Wang, Talanta 41 (1994) 455.
[6] H. Kotte, B. Grundig, K.D. Vorlop, B. Strehlitz, U. Stottmeister, Anal. Chem. 67 (1995) 65.
[7] K.M. Rogers, Biosens. Bioelectron. 10 (1995) 533.
[8] P. Onnerfjord, J. Emneus, G. Marko-Varga, L. Gorton, F. Ortega, E. Dominguez, Biosens. Bioelectron. 10 (1995) 607.
[9] S. Cosnier, Electroanalysis 9 (1997) 894.
[10] L. Campanella, T. Beone, M. Sammartino, M. Tomasseti, Talanta 41 (1994) 1015.
[11] D. Wilcox, A.G. Porras, Y.T. Hwang, K. Lerch, M.E. Winkler, E.I. Solomon, J. Am. Chem. Soc. 107 (1985) 4015.
[12] J. Peisach, P. Aisen, W.E. Blumberg (Eds.), The Biochemistry of Copper, Academic Press, New York, 1966, p. 64.
[13] R.D. Bourbonnais, D. Rochefort, M. Paice, S. Renaud, D. Leech, TAPPI J. 80-68 (2000) 357.
[14] G.F. Hall, D.J. Best, A.P.F. Turner, Anal. Chim. Acta. 213 (1988) 113.

[15] I. Willner, E. Katz, B. Willner, Electroanalysis 9 (1997) 965.
[16] M.P. Connor, J. Sanchez, Analyst 114 (1989) 1427.
[17] J. Kulys, R.D. Schmid, Anal. Lett. 23 (1990) 589.
[18] J. Wang, K. Varughese, Anal. Chem. 62 (1990) 318.
[19] P. Skládal, Collect. Czech. Chem. Commun. 56 (1991) 1427.
[20] F. Botrè, F. Mazzai, M. Lanzi, G. Lorenti, C. Botrè, Anal. Chim. Acta 255 (1991) 59.
[21] F. Ortega, E. Dominguez, J. Biotechnol. 31 (1993) 289.
[22] J. Wang, Y. Lin, Anal. Chim. Acta 271 (1993) 53.
[23] S. Uchiyama, Y. Hasebe, H. Shimizu, H. Ishihara, Anal. Chim. Acta 276 (1993) 341.
[24] L. Campanella, A. Fortuney, M.P. Sammartino, M. Tomasseti, Talanta 41 (1994) 1397.
[25] S. Naish-Byfield, C.J. Cooksey, P.A. Riley, Biochem. J. 304 (1994) 155.
[26] M.J. Dennison, J.M. Hall, A.P.F. Turner, Anal. Chem. 67 (1995) 3922.
[27] Q. Deng, S. Dong, Anal. Chem. 67 (1995) 1357.
[28] C. Petit, A.G. Cortes, J.M. Kauffmann, Talanta 24 (1995) 1783.
[29] G.M. Varga, E. Burestedt, C.J. Svensson, J. Emnéus, L. Gorton, T. Ruzgas, Electroanalysis 8 (1996) 1121.
[30] D. Puig, T. Ruzgas, J. Emnéus, L.O. Gorton, G.M. Varga, D. Barcelo, Electroanalysis 8 (1996) 885.
[31] E.I. Iwuoha, M.R. Smyth, Biosens. Bioelectron. 12 (1997) 53.
[32] F. Trudeau, F. Daigle, D. Leech, Anal. Chem. 69 (1997) 882.
[33] M.W. Jung, K. Kim, J.S. Rhee, J.T. Park, K.J. Paeng, Microchem. J. 70 (2001) 123.
[34] S.M. Lim, H.J. Chung, C. Heete, W.Y. Lee, K.J. Paeng, Anal. Chim. Acta 453 (2002) 81.
[35] M.H. Smit, G.A. Rechnitz, Electroanalysis 5 (1993) 747.
[36] G. Turdean, I.C. Popescu, L. Onicin, D.R. Thevenot, Enzyme Inhib. 17 (2002) 107.
[37] G. Robinson, D. Leech, M.R. Smyth, Electroanalysis 7 (1995) 952.
[38] J. Wang, L. Chen, Anal. Chem. 67 (1995) 3824.
[39] L. Stancik, L. Macholán, F. Scheller, Electroanalysis 7 (1995) 649.
[40] Y. Hasebe, K. Oshima, O. Takise, S. Uchiyama, Talanta 42 (1995) 2079.
[41] K. Streffer, H. Katz, C.B. Bauer, A. Makower, T.H. Schulmeister, F.W. Scheller, M.G. Peter, U. Wollenberger, Anal. Chim. Acta 362 (1998) 81.
[42] M.H. Smit, G.A. Rechnitz, Anal. Chem. 65 (1993) 380.
[43] D.A. Roston, P.T. Kissinger, Anal. Chem. 53 (1981) 1695.
[44] D.V. Roberts (Ed.), Enzyme Kinetics, Cambridge University Press, Cambridge, 1977, p. 153.
[45] H. Ju, D. Zhou, Y.I. Xiao, H. Chen, Electroanalysis 10 (1998) 541.
[46] A.E. Cass (Ed.), Biosensors. A Practical Approach, IRL Press, Oxford, 1990, p. 378.
[47] S.E. Stanca, I.C. Popescu, L. Oniciu, Talanta 61 (2003) 501.
[48] L. Gorton, Electroanalysis 7 (1995) 23.
[49] S. Cosnier, I.C. Popescu, Anal. Chim. Acta 319 (1996) 345.