Biosensors and Bioelectronics Open Access

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Electrochemical Studies on the Kinetic Behaviour of Some Immobilized Enzymes

Margarita Stoytcheva[1*], Roumen Zlatev[1], Zdravka Velkova[2], Velizar Gochev[3], Gisela Montero[1], Benjamin Valdez[1]

[1]Universidad Autónoma de Baja California, Instituto de Ingeniería, Mexicali, México

[2]Medical University of Plovdiv, Department of Chemical Sciences, Plovdiv, Bulgaria

[3]University of Plovdiv "P. Hilendarski", Department of Biochemistry and Microbiology, Plovdi, Bulgaria

[*]**Corresponding author:** Margarita Stoytcheva, Universidad Autónoma de Baja California, Instituto de Ingeniería, Blvd. B. Juárez s/n, Mexicali, México. Tel: +526865664150; Fax: +526865664150; Email: margarita.stoytcheva@uabc.edu.mx

Abstract

The immobilized enzymes are extensively used in the industrial and analytical practices. The efficiency of the processes they catalyse is highly dependent on their kinetic behaviour, which differs from that of the free enzymes and which is not fully investigated. Hence, the objective of this work was to evaluate the main kinetic parameters of the reactions, catalysed by two immobilized enzymes: tyrosinase and organophosphorus hydrolase. These enzymes are frequently used in electrochemical biosensors for environmental pollutants determination. An appropriate electrochemical approach was applied, performing the measurements under enzyme kinetics control and analysing the results in accordance to the model of Shu and Wilson.

It was demonstrated that catechol (K_M^{app}= 5.46 mmol L^{-1}) presents greater affinity to the immobilized tyrosinase, than phenol (K_M^{app}=9.65 mmol L^{-1}). The affinity to the immobilized organophosphorus hydrolase of its substrates decreased in the order: methyl parathion (K_M^{app}=248.12 µmol L^{-1}), parathion (K_M^{app}=250.92 µmol L^{-1}), paraoxon (K_M^{app}= 425.08 µmol L^{-1}). The sensitivity of the quantitative determinations increased with K_M^{app} decrease. The presented work provides new and additional information on the catalytic properties and kinetic behaviour of the immobilized enzymes tyrosinase and organophosphorus hydrolase, especially.

Introduction

The immobilized enzymes are defined as "Enzymes physically confined at or localized in a certain region or space with retention of their catalytic activity and which can be used repeatedly and continuously" [1]. Enzyme immobilization offers such significant advantages as extended use due to enzyme stabilization, localization of the interaction, efficient control of the reaction parameters, and prevention of the products contamination, among other [2]. Owing these characteristics, the immobilized enzymes found numerous industrial and analytical applications [3-8]. However, enzyme structure modifications during enzyme immobilization, as well as the appearance of steric, partition, micro-environmental, and diffusion hindrances affect the kinetic properties of the enzyme reactions [9].

The kinetic behaviour of the immobilized enzymes used as a recognition element in the electrochemical biosensors was firstly theoretically described by Blaedel et al. [10], which consider a simple idealized enzyme system. The model they suggested was validated treating the potentiometric response of a urease electrode. The catalytic properties of the immobilized enzymes as a part of the stationary amperometric sensors were modelled by Mell and Maloy [11]. They predict the steady-state current response of the biosensor applying digital simulation under kinetic and diffusion reaction control, respectively. Nevertheless, since the boundary conditions to describe the mass transport to stationary electrodes are not well defined, their application to investigate the kinetic behaviour of the immobilized enzymes is limited. More noteworthy kinetic model was suggested by Shu and Wilson [12], taking advantage of the well-known convection diffusion behaviour of the rotating disk electrode. Nowadays, the convection diffusion equation (Eq. 1) and the enzyme kinetic equation (Eq. 2) they

propose are commonly used to describe the kinetics of the enzyme reactions applying electrochemical biosensors:

$$I_s = 0.65 z F A D^{2/3} v^{-1/6} \omega^{1/2} [S] \qquad (1)$$

$$\frac{I_{max}}{I_s} = \frac{K_M}{[S]} + 1 \qquad (2)$$

Eq. (1) is identical to the Levich equation for the steady-state current of the rotating disk electrodes [13]. I_s is the steady-state current, z is the number of exchanged electrons, F is the Faraday constant, A is the geometrical area of the electrode, D is the diffusion coefficient, v is the kinematic viscosity of the solution, ω is the angular electrode rotation rate, and $[S]$ is the substrate concentration.

Eq. (2) is similar to the Michaelis-Menten equation [14]. I_{max} is the maximal steady-state current, corresponding to the maximal velocity of the enzyme reaction, and K_M is the Michaelis-Menten constant. As these parameters differ from those established in homogeneous phase, they should be referred as "apparent". The goal of this work is to investigate the kinetic behaviour of two immobilized enzymes: tyrosinase (Tyr) and Organophosphorus Hydrolase (OPH), which are frequently used in electrochemical biosensors for environmental pollutants determination, applying an appropriate electrochemical approach.

Experimental

Reagents

All the substances were of analytical reagent grade and were used as such, without further purification. Tyrosinase substrates (phenol and catechol), as well as organophosphorus hydrolase substrates (paraoxon ethyl, parathion, and methyl parathion), were purchased from Sigma. Solutions of phenol, catechol, and paraoxon ethyl were prepared in deionized water. Parathion and methyl parathion were dissolved in methanol. 0.2% w/v solution of chitosan (medium molecular weight, Sigma) was obtained by dissolving the substance in CH_3COOH 0.1 mol L^{-1}. Britton-Robinson buffer (H_3PO_4 0.04 mol L^{-1}, CH_3COOH 0.04 mol L^{-1}, H_3BO_3 0.04 mol L^{-1}, and NaOH 0.2 mol L^{-1}) with pH 6.5 (corresponding to Tyr pH optimum) and 8.5 (corresponding to OPH pH optimum) was used as a supporting electrolyte. Tyrosinase (EC 1.14.18.1) was provided by Spectrum Chemical, USA. Enzyme activity unit was defined as the amount of enzyme that liberates 1 μmol of o-quinone per minute from catechol under the assay's conditions. The specific enzyme activity was found to be 224 units per mg. Organophosphorus hydrolase (EC 3.1.8.1) was extracted from the decontamination product DEFENZ™ 130BG, supplied by Genencor International, Inc. The enzyme activity unit was defined as the amount of enzyme that liberates 1 μmol of p-nitrophenol from paraoxon per minute under the assay's

conditions. The specific enzyme activity was found to be 54 units per mg. The measurements for enzyme activity evaluation were performed applying spectrophotometric techniques, and using a PC controlled Evolution 60S UV-VIS spectrophotometer.

Electrochemical Instrumentation and Procedures

The electrochemical analyser CH Instruments model 440 A, equipped with a 3-electrode electrolysis cell of conventional type was used to perform the electrochemical measurements. The 3-electrodes system included a working electrode elaborated from spectrally pure graphite (Ringsdorf Werke, Germany, 3 mm diameter), a Pt wire as an auxiliary electrode, and an Ag, AgCl/KCl$_{sat}$ electrode as a reference. The working electrode, after the common procedures of polishing, degreasing, and cleaning was modified according to the following protocol: spin-coating onto the electrode surface of 5 μL of a blend of chitosan and enzyme solution in 1:1 ratio; hydrogel film formation for 60 min at ambient temperature; chitosan cross-linking by dropping onto the film surface of 5 μL of glutaraldehyde 2.5% and allowing it to react for 10 min; rinsing the modified electrode with PBS to remove the excess of glutaraldehyde; a second enzyme-entrapped chitosan layer formation following the same procedure [15,16]. The electrochemical method selected to achieve the measurements was pulsed amperometric detection, which is very efficient in avoiding electrode fouling. The optimized measuring parameters values are listed in Table 1 [15,16].

Parameters	Tyr sensor	OPH sensor
E_d, V vs. Ag, AgCl/KCl$_{sat}$	0.00	+1.10
τ_d, ms	66.64	66.64
E_{cl}, V vs. Ag, AgCl/KCl$_{sat}$	+1.40	+1.40
τ_{cl}, ms	166.6	166.6

Table 1: Parameters values for the pulsed amperometric detection of phenols and organophosphorus compounds using respectively Tyr- and OPH-based electrodes. E_d is the detection potential; τ_d is the detection time; E_{cl} is the cleaning potential; τ_{cl} is the cleaning time.

All the measurements were performed at ambient temperature and at pH corresponding to the enzyme pH optimum. The rotation speed of 700 rpm of the working electrode guaranteed the fast mass transport and allowed eliminating the diffusion constraints. Thus, the investigated processes were controlled by the kinetics of the enzyme reactions.
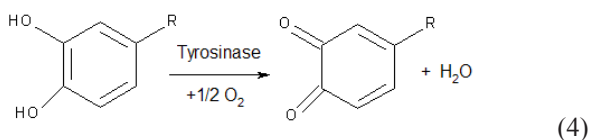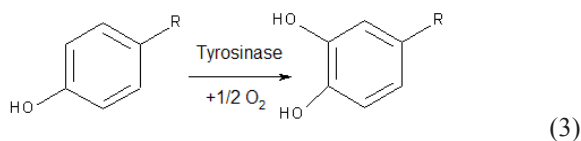
Data Treatment

All the presented data are the average of at least 5 measurements. The kinetic parameters of the enzyme reactions were assessed by means of the statistical software package GraphPad Prism (GraphPad Software, San Diego, California, USA, www.graphpad.com), applying Michaelis-Menten nonlinear regression. The calibration plots were obtained by fitting the model $I_s = I_{max}[S]/$
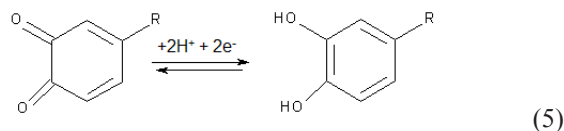
$(K_M+[S])$ to the obtained data.

Results and Discussion

Kinetic Behaviour of the Immobilized Tyrosinase

Tyrosinases (Tyr: E.C. 1.14.18.1) are copper-containing oxidases, which catalyse the o-hydroxylation of monophenol molecules to o-diphenols (Eq. 3), as well as the oxidation of the o-diphenols to o-quinones (Eq. 4):

$$\text{(3)}$$

$$\text{(4)}$$

The amperometric quantification of phenolic compounds applying a tyrosinase sensor is based on the measurement of the current of the o-quinones reduction (Eq. 5), which is proportional to the enzyme substrate concentration.

$$\text{(5)}$$

The obtained amperometric curves and the corresponding calibration plots for phenol and catechol determination applying the described measuring principle are presented in Figure 1.
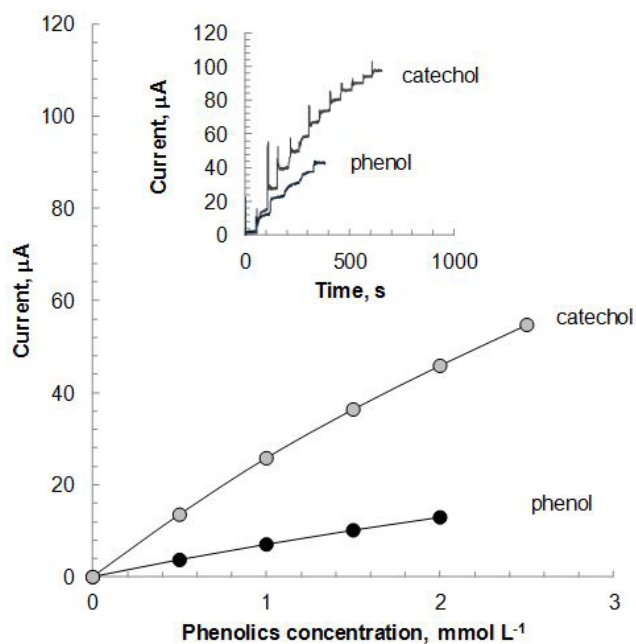

Figure 1: Amperometric curves and the corresponding calibration plots for phenol and catechol determination applying a tyrosinase-based electrochemical sensor.

The amperometric response of the sensor was analysed according to the model of Shu and Wilson [12]. The linearization of the electrode transfer function (Eq. 2) was carried out by the method of Hanes-Woolf [17], as shown in Figure 2. This graphical presentation is considered as the most accurate among the other currently used graphical approaches allowing determining the Michaelis-Menten constant and the maximum velocity of the enzyme catalysed reactions.
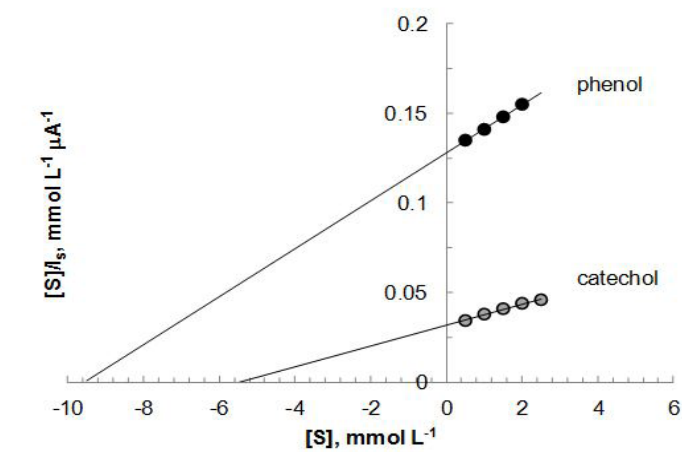
Figure 2: Hanes-Woolf linearization of the electrode transfer function of the tyrosinase sensor

The kinetic parameters: maximal velocity of the tyrosinase catalysed reactions of phenol and catechol conversion, and the apparent Michaelis-Menten constants, were derived from the slope of the lines and the x-intercepts, which equal $1/I_{max}$ and $-K_M^{app}$, respectively. The value of the Michaelis-Menten constant for the immobilized tyrosinase was found to be 5.46 mmol L^{-1} in the presence of catechol, and 9.65 mmol L^{-1} in the presence of phenol. These values indicate the higher affinity of the immobilized tyrosinase to catechol, and were reflected on the sensitivity of the phenolics determination. It increases with K_M^{app} decrease. The sensitivity of catechol determination was found to be 22.94 μA mmol^{-1} L, (R^2=0.9889) and it is higher than the sensitivity of phenol determination, which equals 7.10 μA mmol^{-1} L (R^2=0.9980). The order of sensitivity decrease is in conformity with the reported in the literature [18-24].

It is important to note that the values of the apparent Michaelis-Menten constant of the immobilized tyrosinase vary in a very large interval, ranging from 8.9 μmol L^{-1} to 54.9 mmol L^{-1}, and from 6.6 μmol L^{-1} to 0.2 mol L^{-1}, using correspondingly phenol and catechol as substrates (Table 2). The value of K_M^{app} is obviously strongly dependant on the immobilization matrix and the applied immobilization procedure.

Matrix	K_M^{app}, mmol L^{-1} (phenol as substrate)	K_M^{app}, mmol L^{-1} (catechol as substrate)	Ref.
Tyr-chitosan/laponite-GCE	0.460	0.160	[19]
Tyr-poly(dicarbazole)-Pt	-	0.800	[25]
Tyr-gelatin-GCE	0.039	0.157	[24]
Tyr-agarose/guar gum-GCE	-	0.022	[26]
Tyr-AuNP-GCE	0.140	0.120	[27]
Tyr-Au$_{coll}$-CPE	0.0536	0.0485	[28]
Tyr-CPE	0.0712	-	[28]
Tyr-ZnO/chitosan-GCE	0.023	0.040	[29]
Tyr-Au$_{coll}$-graphite/Teflon	0.0089	0.0066	[30]
Tyr-MWCNT/ZnO/Nafion-GCE	0.018	0.020	[20]
Tyr-MWCNT/IL/DHP-GCE	-	0.190	[31]
Tyr-PPy-Pt	-	100.00	[32]
Tyr-PPy/thiophen-MM-Pt	-	200.00	[32]
Tyr-Au/PASE-GO-SPE	-	0.027	[22]
Tyr-PANI/SWCNT-GCE	-	0.02471	[33]

Table 2: Reported in the literature values of K_M^{app} of the immobilized tyrosinase in electrochemical biosensors for phenolics determination. (CPE-carbon paste electrode; DHP- dihexadecylphosphate; GCE-glassy carbon electrode; GO-graphene oxide; IL-ionic liquid; MM-menthyl monomer; MWCNT-multi-wall carbon nanotubes; NP-nanoparticles; PASE- 1-pyrenebutanoic acid, succinimidyl ester; PANI-polyaniline; PPy-polypyrrole; SWCNT-single-wall carbon nanotubes).
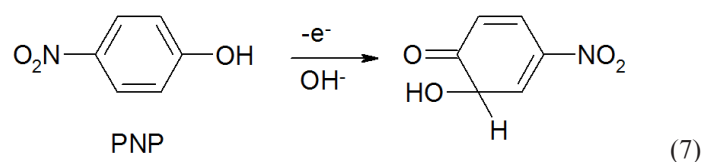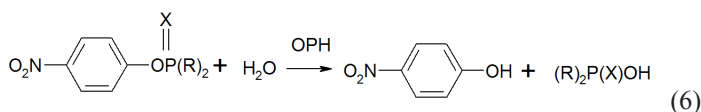
The maximal velocity of the phenol and catechol tyrosinase catalyzed conversion was established to be 74.62 μA and 172, 41 μA, respectively.

Kinetic Behaviour of the Immobilized Organophosphorus Hydrolase

The organophosphorus hydrolase (OPH: EC 3.1.8, also known as aryldialkylphosphatase, phosphotriesterase, or paraoxon hydrolase) represents an interest because of its activity on warfare agents and pesticides. It's typical substrates are the organophosphorus pesticides paraoxon, parathion, methyl parathion, malathion, coumaphos, and acephate, and the nerve agents VX, R-VX, DFF, sarin, soman, and tabun, among other. As the enzyme catalyses the hydrolysis of the P-O, P-S, P-F, and P-CN bonds, it is used in some decontamination formulations, as well as in electrochemical biosensors for environmental pollution control and detoxification processes monitoring.

The kinetic behaviour of the free OPH is commented in the literature [34-36]. Nevertheless, only few data are reported on the kinetic activity of the immobilized OPH [37,38]. Hence, results presented in this work will provide new valuable information.

The performed measurements were based on the amperometric quantification of the Organophosphorus Pesticides (OP) paraoxon, parathion, and methyl parathion applying an OPH-sensor. The enzyme catalyses the OP hydrolysis, yielding P-Nitrophenol (PNP) (Eq. 6). The analytical signal is the current of PNP oxidation, which is proportional to the OP concentration (Eq. 7).

$$O_2N-\text{C}_6\text{H}_4-OP(R)_2 + H_2O \xrightarrow{OPH} O_2N-\text{C}_6\text{H}_4-OH + (R)_2P(X)OH \quad (6)$$

$$O_2N-\text{C}_6\text{H}_4-OH \xrightarrow[OH^-]{-e^-} \quad (7)$$

PNP

X is oxygen or sulphur; R is an alcoxy group (methoxy to butoxy). The recorded amperometric curve for paraoxon as an example, and the constructed calibration plots for paraoxon, parathion, and methyl parathion determination are presented in Figure 3.
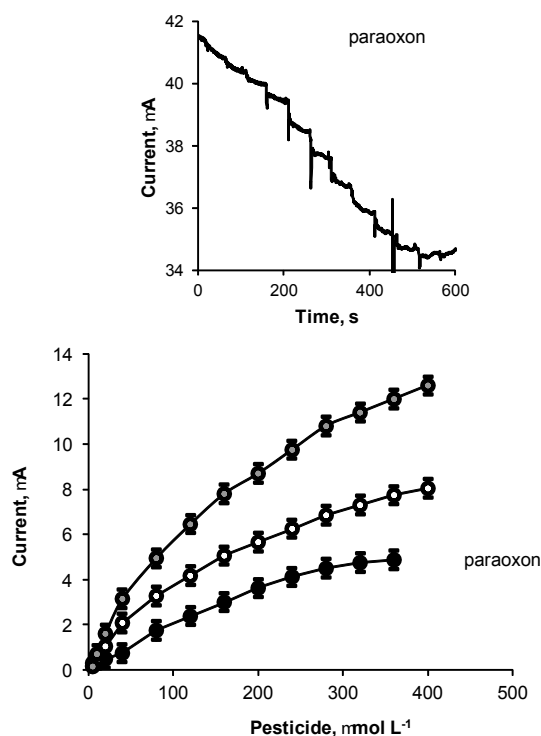

Figure 3: Amperometric curve for paraoxon, and the constructed calibration plots for paraoxon, parathion, and methyl parathion, applying an OPH-based sensor.

Data were analysed as described above. The obtained Hanes-Woolf plots are displayed in Figure 4. The lower K_M^{app} value found is 248.12 $\mu mol \, L^{-1}$, when using methyl parathion as OPH substrate. The K_M^{app} value established in the presence of parathion was equal to 250.92 $\mu mol \, L^{-1}$, i.e. close to the previous. The K_M^{app} value was the highest (425.08 $\mu mol \, L^{-1}$), when the substrate used was paraoxon. The maximal velocity of the enzyme reaction was found to be: 11.05 μA (paraoxon), 13.02 μA (parathion), and 20.16 μA (methyl parathion).
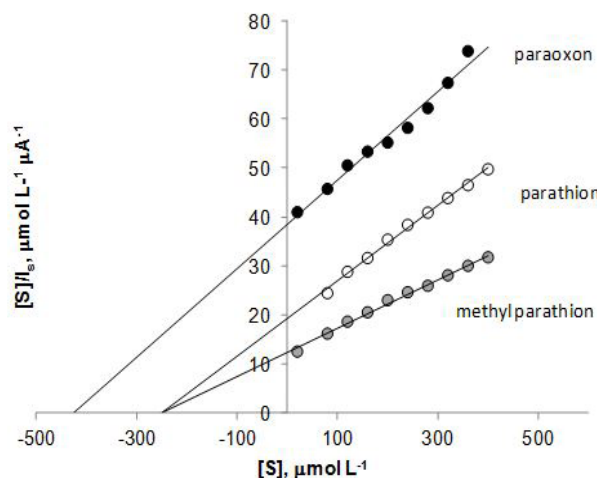

Figure 4: Hanes-Woolf linearization of the electrode transfer function of the OPH sensor.

Obviously, OPH demonstrates greater affinity to parathion and methyl parathion. The enzyme used in this work was extracted from the decontamination formulation DEFENZ™ 130BG. According to the producer, the organophosphorus hydrolase included has demonstrated activity against synthetic substances that mimic the breakdown of VX, Russian-VX, and pesticides such as parathion.

Obtained data have an analytical importance too, as the sensitivity of the OPs determination applying an OPH electrochemical sensor increases in the range: paraoxon>parathion>methyl parathion, correspondingly to the decrease of the K_M^{app} values. The sensitivity was evaluated to be: 22.2 nA L μmol^{-1}, 51,9 nA L μmol^{-1}, 60.8 nA L μmol^{-1}, respectively.

Conclusion

The kinetic behaviour of the immobilized enzymes tyrosinase and organophosphorus hydrolase was investigated using electrochemical biosensors and applying pulsed amperometric detection under enzyme kinetics control of the processes. The values of the main kinetic parameters K_M^{app} and maximal velocity of the enzyme reactions were calculated, thus providing new and additional information on the properties of the immobilized enzymes. Such data could be helpful in improving the performances of the electrochemical biosensors.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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