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Direct monitoring of pollutants based on an electrochemical biosensor with novel peroxidase (POX_{1B})

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ABSTRACT

A biosensor for the monitoring of phenolic compounds based on a new protein named POX_{1B} purified from garlic which demonstrates similar biochemical properties to peroxidase is investigated. The enzyme was immobilized into chitosan microspheres with covalent link. The properties of the biosensor were analyzed with Fourier transform-infrared spectroscopy (FT-IR), scanning electron microscopy (SEM) and cyclic voltammetry (CV). FT-IR demonstrates the covalent attachment of POX_{1B} into chitosan and SEM shows high dispersion of the POX_{1B} into the chitosan microspheres. The redox potential of POX_{1B} in chitosan is 147 mV *vs*. SCE, which is much higher than reported works using HRP, demonstrating excellent direct electrochemical behaviour of the POX_{1B}. The electrocatalytic activity of the obtained biosensor towards chlorophenols derivatives in a large range from 10 pM to 10 μM was demonstrated. The mediator free POX_{1B}-based biosensor exhibited high sensitivity towards 2,6-dichlorophenol, 4-chlorophenol and pentachlorophenol. A detection limit of 1 pM in the case of 4-chlorophenol was demonstrated with kinetic constant $K_{m,app}$ of 0.42 μM with high rapidity of electrochemical response of the biosensor of 1 s.

1. Introduction

Phenolic contaminants, mainly chlorophenols, are harmful substances found in the wastewaters of several industries such as coal conversion, petroleum refining, resins and plastics, wood preservation, metal coating, dyes and other chemicals (Nicell et al., 1993). They occur at concentrations typically ranging from 100 to 1000 mg L^{-1} (Faust and Aly, 1983). These substances are classified as toxic and persistent in the environment and hence heavily regulated in many countries (Gellman, 1988; Garden, 1990). The Economic European Community (EEC) in its directive 76/464/CEE has classified phenols including 2-amino-4-chlorophenol, 4-chloro-3-methylphenol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, pentachlorophenol and trichlorophenol as dangerous substances discharged into the aquatic environment (Vincent, 1991). The U.S. Environmental protection Agency (EPA) lists 11 other phenolic compounds which includes 2,6-dichlorophenol (Keith and Telliard, 1979). Synthetic phenols are more toxic than the natural ones and public concern and legislation are nowadays demanding better environmental control. As a consequence, development of rapid and reliable tools for the detection of phenolic compounds is of great interest.

The conventional spectrophotometric and chromatographic methods for the determination of phenols in waters are time-consuming and do not easily allow continuous monitoring in real samples (Hanraham et al., 2004). Hence, there is a pressing need for the development of an accurate sensor for these compounds. Several biosensors involving enzymes have been developed for phenolic compounds monitoring. Most of them are based on various tyrosinases provided from mushroom (Ortega et al., 1994; Yaropolov et al., 1995), banana (Wang and Lin, 1989) or potato (Wang and Naser, 1992). Biosensors based on other enzymes such as lignin peroxidase (Fawer et al., 1991) or flavin monooxygenase (Neujahr and Kjellen, 1979) have also been investigated. Tyrosinase biosensors are restricted to the monitoring of relatively few phenolic compounds. Peroxidases are oxidoreductase proteins produced by a number of microorganisms and plants. They can be used in biosensing devices for the determination of phenols based on their double displacement or "ping-pong" mechanism in which two substrates—hydrogen peroxide and the given phenolic compound are involved (Anni and Yonetani, 1992; Ryan et al., 1994). However, these devices do not detect low concentrations of phenolic compounds, which remains a challenge and necessary for close monitoring of their cumulative effects on the environment. Hence, new proteins with high stability and activity are needed for the development of biosensor devices.

With this aim, a new protein named (POX_{1B}) purified from garlic bulb (*Allium sativum* L.) was recently produced by our group and some of their biochemical properties were investigated showing

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similar behaviour to peroxidase (El Ichi et al., 2008). Biochemical characterization of this isoform revealed that it has great potential in application for biosensors design. In fact, an excellent sensitivity was obtained when POX_{1B} was used for hydrogen peroxide monitoring. The detection limit attained 100 nM of hydrogen peroxide in direct detection mode without any labelling or amplification step which allowed the POX_{1B}-based biosensor to control fraud in real samples such as milk (El Ichi et al., 2009). The use of a POX_{1B}-based biosensor in monitoring chlorophenols aims to show the versatility of this novel enzyme. In the present work, chitosan microspheres cross-linked with glyoxal were used for immobilizing peroxidase POX_{1B} on the gold electrode surface. This polymer has good film-forming ability, chemical inertness, high mechanical strength, high hydrophilic and biocompatibility (Wu et al., 2001) which makes it a suitable immobilization matrix for enhancing the stability of the POX_{1B}-based biosensor. The glyoxal as linking agent should allow a higher stability and non-denaturizing of the enzyme than carbodiimide and glutaraldehyde which are generally used for covalent linkage (Munteanu et al., 1998). In this work, we have developed a new biosensor involving POX_{1B} immobilized into chitosan microspheres by covalent attachment and characterized by FT-IR, SEM and cyclic voltammetry. This biosensor was tested for the detection of some pollutants such as chlorophenol derivatives through the biocatalysis reactions obtained by POX_{1B} and by the electrochemical detection of intermediate compounds.

2. Experiments

2.1. Reagents

Peroxidase POX_{1B} was purified and biochemically characterized in a preceding work (Medjeldi Marzouki et al., 2005; El Ichi et al., 2008). This enzyme is highly active at pH 5 and at 30 °C. It is stable with temperature and storage in its free and immobilized form (El Ichi et al., 2009). Chitosan (from crab shells) and Glyoxal trimer dihydrate were purchased from Sigma–Aldrich (www.sigma-aldrich.com). All reagents were of the highest purity and all the solutions were prepared with deionized water.

2.2. Preparation of the POX_{1B}-based electrode

Chitosan microspheres were prepared according to published procedures (Denkbaş and Odabaşi, 2000) including some modifications to obtain highly dispersive microspheres. A 1% chitosan solution was prepared in 2% warm acetic acid solution, then added dropwise to a dispersion medium containing mineral oil, petroleum ether (0.5/0.7, v/v) and 0.8 mL Tween as emulsifier. The mixture was mechanically stirred in the range of 1000–2000 rpm at room temperature. After 10 min, 0.02 mL of glutaraldehyde was added into the dispersion medium. Similarly, 1 h later, a further 0.02 mL of glutaraldehyde was added to the solution and then stirred for 2 h. Chitosan microspheres were collected by centrifugation and washed with petroleum ether, sodium bisulfide, and acetone consecutively. The obtained microspheres were dried in an oven at 40 °C for 2 days and kept in a vacuum dessicator until further use. The immobilization of the protein into chitosan microspheres was realized following the procedure described by Zubriene and collaborators (2003) for the immobilization of hydrolases. A reaction mixture containing 0.2 g (dry weight) of chitosan microspheres, 0.8 mL of 0.01 mol L^{-1} PBS pH 7.4 and 0.4 mL from the enzymatic solution (15 U/mL) was incubated for 1 h at 30 °C under stirring. Then, the chitosan microspheres with immobilized protein were filtered and rinsed with 1 mL 0.01 mol L^{-1} PBS pH 7.4 and conserved in a minimum volume of buffer. Before the immobilization on the electrode surface, 15 μL of the suspension of the chitosan/POX_{1B} was taken from a reaction mixture and 5 μL glyoxal (0.05%) was added which plays the role of cross-linker of the chitosan microspheres. A low concentration of glyoxal was added to avoid the denaturizing of the POX_{1B}. 10 μL of the mixture was then deposited on the surface of the electrode. The biosensor was stored overnight at 4 °C in dry conditions. Once used, it is stored at 4 °C in 0.01 mol L^{-1} PBS pH 7.4.

2.3. Fourier transform-infrared spectroscopy (FT-IR)

A Brucker IFS 66 spectrometer with MCT detector was used to acquire infrared spectra of chitosan microspheres and POX_{1B} immobilized into chitosan matrix.

2.4. Scanning electron microscopy (SEM)

SEM images were acquired using a ZEISS SUPRA™ 55VP GEMINI® apparatus. Chitosan microparticles and immobilized POX_{1B} into chitosan were prepared in deionized water. Thin film samples were prepared by spin coating on glass substrates. Scans were performed in 2–20 μm range.

2.5. Cyclic voltammetry and chrono amperometry assays

Cyclic voltammetry and chrono amperometry experiments were performed in a three-electrode cell using a gold electrode as the working electrode, a platinum-mesh as the counter electrode, and a saturated calomel electrode (SCE) as the reference electrode. The buffer solution was thoroughly deoxygenated using oxygen-free argon prior to the electrochemical measurements. An Autolab PGSTA 100 potentiostat controlled by GPES Autolab software was used for the electrochemical measurements. Cyclic voltammetry measurements were achieved at the scan rate of 50 mV s^{-1} and at room temperature. The sweep potential was from −0.5 to +0.6 V. Three negative controls were assayed in presence of 0.01 mol L^{-1} PBS pH 7.4, 1×10^{-3} mol L^{-1} H_2O_2 and 1×10^{-3} mol L^{-1} 2,6-dichlorophenol respectively. The applied potential for chrono amperometry assays was −0.35 *vs*. SCE.

3. Results and discussions

3.1. Biosensor based on immobilized POX_{1B} into chitosan microparticles

POX_{1B} isoenzyme was purified from garlic bulb (*Allium sativum* L.) by ammonium sulfate precipitation, gel filtration and anion exchange chromatography. Biochemical properties of this new protein were previously characterized.

Previous studies on the biochemical properties of this new protein showed that it is highly active in acidic media and thermally stable which are characteristics that can be exploited in biosensing applications (El Ichi et al., 2008). The immobilization of the POX_{1B} was performed by entrapping the enzyme into chitosan microspheres prepared by a new procedure. Chitosan dissolved in acetic acid solution was dispersed, using high stirring rates, in a mixture of mineral oil, petroleum ether and Tween as emulsifier. This procedure allows obtaining the small size and high distribution of chitosan microspheres. The POX_{1B} was immobilized by covalent link into chitosan microspheres. A low concentration of glyoxal was used as cross-linker of chitosan microspheres instead of glutaraldehyde to avoid enzyme denaturizing before their casting on the surface of gold electrode. Scanning electronic microscopy (SEM) was investigated to show the morphologies of the surface layer before and after POX_{1B} immobilization on the chitosan microspheres (Fig. 1). The images show that chitosan microparticles are

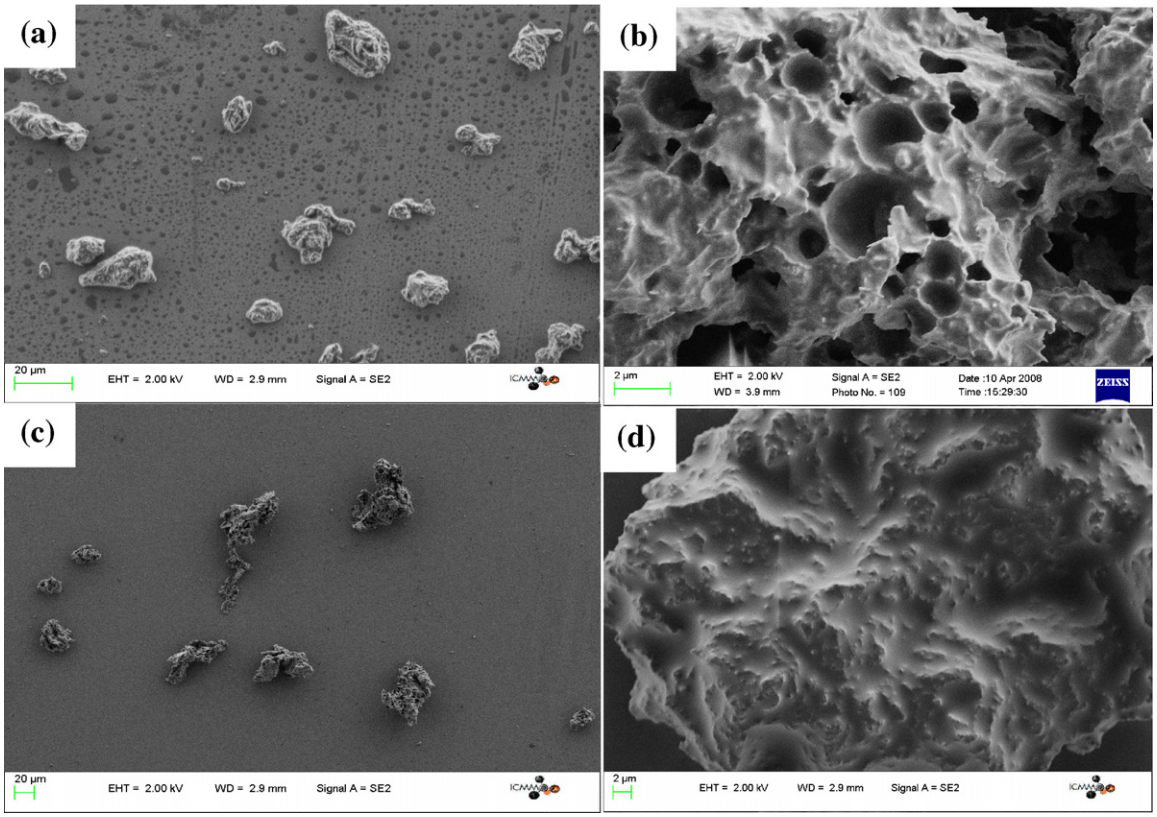

Fig. 1. SEM images of chitosan (a and b) and peroxidase POX_{1B} into chitosan (c and d).

not completely spherical (Fig. 1a) and the porous structure of chitosan is apparent (Fig. 1b). This finding agrees with previous studies reported in the literature (Denkbaş and Odabaşi, 2000). The size of chitosan microparticles ranged mainly from 10 to 20 μm and the size of apparent pores ranged from 0.8 to 3 μm. The chitosan microspheres size reported in the literature is in the range of 30–800 μm (Denkbaş and Odabaşi, 2000; Zubriene et al., 2003). The morphology and size distribution of the chitosan microspheres depends on the swelling behaviour of chitosan. In our case, smaller chitosan microparticles were obtained by using a lower chitosan/acetic acid solution ratio and applying higher stirring rates than those reported by Denkbaş and Odabaşi (2000). Using smaller and narrower chitosan microspheres allows gold electrode coating with a thin and uniform polymer layer. The immobilized POX_{1B} into chitosan induces a disappearance of the porous structure of chitosan (Fig. 1c) which confirms the proteins attachment.

To characterize the obtained chitosan microspheres and to confirm the presence of peroxidase POX_{1B} in the immobilization matrix, Fourier transform-infrared (FT-IR) spectroscopy (Fig. 2) was performed. POX_{1B} isoenzyme, chitosan and chitosan-POX_{1B} were analyzed in the 1200–1800 cm^{-1} range. Fig. 2 shows that in addition to the bands assigned to chitosan (Fig. 2b) obtained at 1026 and 1073 cm^{-1}, the presence of POX_{1B} (Fig. 2c) gives two additional large bands at 1650 and 1550 cm^{-1} corresponding to the amide I and amide II vibrations of the amino acid bond in the protein chain observed for the protein itself (Fig. 2a) (Korri-Youssoufi et al., 2008). The spectrum of the immobilized POX_{1B} (Fig. 2c) shows a shift in the position of amide I and amide II bond in comparison with the free enzyme where amide I is located at 1630 cm^{-1} and amide II at 1547 cm^{-1} (Fig. 2a). This could be explained by a change in protein conformation due to the orientation of the immobilized peroxidase into the chitosan matrix. In fact, information on the secondary structure of polypeptide chain for proteins can be provided by the shape and position of amide I observed in the range of (1600–1700 cm^{-1}) and amide II (1500–1600 cm^{-1}) infrared bands. The amide I absorption arises predominately from protein amide C=O stretching vibrations, while the amide II absorption arises from amide N–H bending vibrations (60%) coupled to the C–N stretching vibrations (40%) (Liu et al., 1996). The amide I bands allows to characterize the secondary structure of proteins (Paris et al., 2005). In the case of POX_{1B} in free form (Fig. 2a) the amide I band is observed at 1630 cm^{-1} and could be related to a β-strand conformation. Immobilized POX_{1B} into chitosan microspheres allows the appearance of an amide I band at 1650 cm^{-1} (Fig. 2c) which could indicates that the protein has an α-helical configuration in immobi-

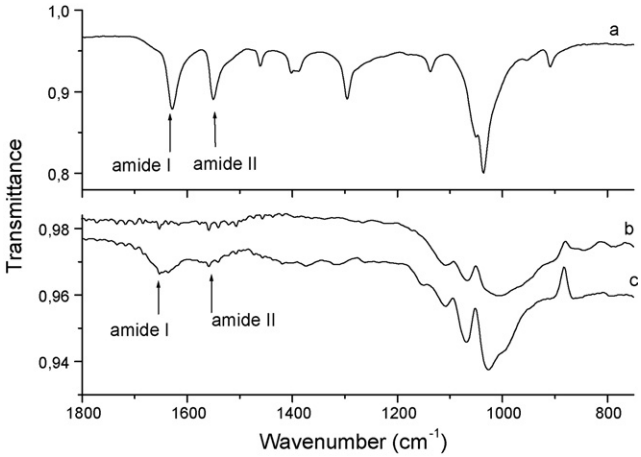

Fig. 2. Infrared spectra of (a) POX_{1B}; (b) chitosan microsphere; (c) POX_{1B} immobilized into chitosan matrix.

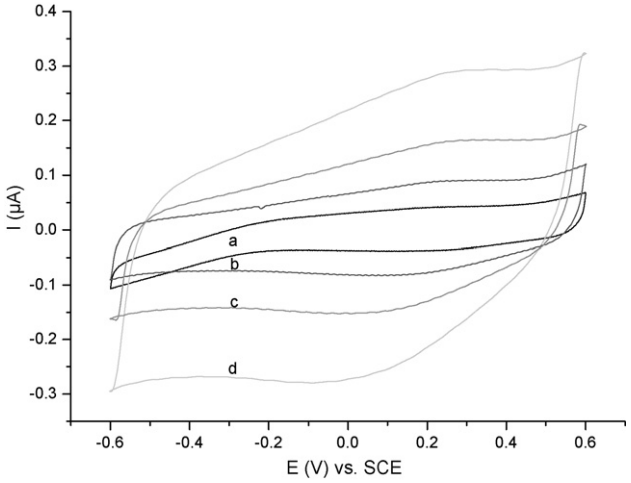

Fig. 3. Cyclic voltammogram of the modified electrode with POX_{1B}-Cs analyzed in 0.01 mol L^{-1} PBS pH 7.4 at different scan rates (mV s^{-1}) (a) 20, (b) 40, (c) 60, (d) 100.

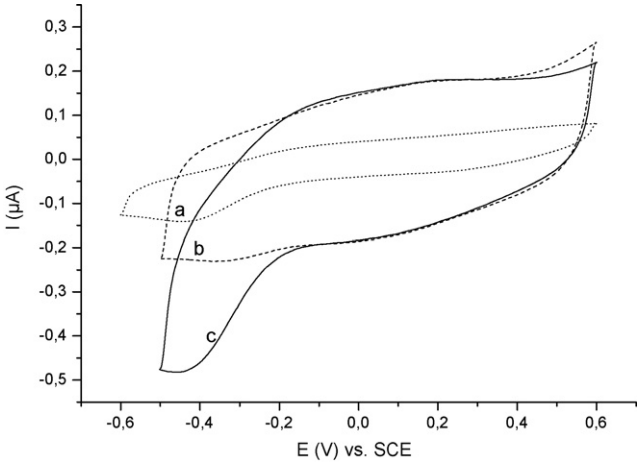

Fig. 4. Electrochemical response of (a) gold electrode coated with chitosan in presence of 0.01 mol L^{-1} PBS pH 7.4, 5×10^{-5} mol L^{-1} H_2O_2 and 1×10^{-3} mol L^{-1} 2,6-dichlocrophenol; (b) modified electrode with POX_{1B}-chitosan in presence of 5×10^{-5} mol L^{-1} H_2O_2, (c) modified electrode with POX_{1B}-chitosan in presence of 5×10^{-5} mol L^{-1} H_2O_2 and 1×10^{-3} mol L^{-1} 2,6-dichlocrophenol.

lized form. More experiments are under investigation to determine the conformation of this new enzyme in free and immobilized form.

Electrochemical activity of the POX_{1B} was investigated by cyclic voltammetry in presence of 0.01 mol L^{-1} PBS solution at pH 7.4 (Fig. 3). The electrochemical waves are broad indicating that the heme of the POX_{1B} immobilized into microspheres of chitosan is subject to different local environments. The redox potential E_0 measured from the oxidation and reduction potential waves is 0.147 V *vs*. SCE (0.387 V *vs*. NHE). This potential is higher than the value reported for HRP immobilized into chitosan on a carbon electrode, where the measured potential was −0.09 V *vs*. NHE (Huang et al., 2002) and nano-particles of HRP immobilized on gold electrode (0.070 V *vs*. NHE at pH 7.00) (Liu et al., 2005) and closer to the reported redox potential of HRP on Nafion-Cysteine/Au (0.257 V *vs*. NHE) (Hong et al., 2007). The measured formal redox potential of the POX_{1B} in PBS solution at pH 7.4 is obtained at 0.3 V *vs*. SCE (0.54 V *vs*. NHE). This potential is also higher than the redox potential of HRP in solution at pH 7.00 (−0.270 V *vs*. NHE) (Harbury, 1957). The higher value of redox potential in the case of POX_{1B} compared to a HRP is related to an enhanced rate of electron transfer due to their heme properties. In fact, it is well known that the redox potential of a protein depends on porphyrin conformation, which is the axial ligand of the heme, and thus on the hydrophobicity of the heme pocket (Bento et al., 2003; Capitanio et al., 2000; Rivas et al., 2005). Thus underline that the heme of POX_{1B} does not have similar properties as the HRP enzyme. The decrease of the formal redox potential in the case of free POX_{1B} form (0.3 V *vs*. SCE) compared to the immobilized enzyme (0.147 V *vs*. SCE) could be attributed to the change in their conformation after immobilization into chitosan as demonstrated below with FT-IR analysis.

The current potential varies linearly with scan rate which underlines that the enzyme is immobilized on the surface. The redox couple observed here had an approximately symmetric peak shape with nearly equal heights of reduction and oxidation waves. Integration of reduction peaks at different scan rates gave nearly constant charge values. These are characteristic of diffusionless, thin-layer electrochemical behaviour (Murray, 1984). This suggests that during the redox process all electroactive heme Fe(III) in the films is converted to heme Fe(II). The amount of immobilized enzyme was calculated from the measured charge flow and normalized by electrode area and the value obtained is 4×10^{-9} moles cm^{-2}. This method of assembling POX_{1B} and chitosan microspheres allows better enzymes immobilization with higher electro activity compared to the immobilized HRP into chitosan where the average quantity of electroactive enzyme is 3.8×10^{-11} moles cm^{-2} (Huang et al., 2002). The size of POX_{1B} allows also the high density of the immobilized enzyme compared to HRP. In fact, the POX_{1B} protein has approximately 30 kDa where the molecular weight of HRP is 40 kDa. A smaller size of POX_{1B} in comparison to HRP could also permit a larger amount of immobilized enzyme. Small protein can access more easily to the polymer pores of chitosan microspheres.

3.2. Analytical parameters of POX_{1B}-based biosensor

In order to test the response of the chitosan/POX_{1B}-based electrode to chlorophenol reaction, the electrochemical measurements were performed in solution containing H_2O_2 in absence and in presence of 1×10^{-6} mol L^{-1} of 2,6-dichlorophenol (Fig. 4b and c). A negative control using an electrode coated with non-modified chitosan and analyzed in solution containing H_2O_2 and 2,6-dichlorophenol showed that there is no change in electrochemical signal. This implies that there is no interaction between chitosan and H_2O_2 or 2,6-dichlorophenol (Fig. 4a). In presence of 2,6-dichlorophenol (Fig. 4c) and H_2O_2 as co-substrate a new peak appears at a potential of −0.4 V *vs*. SCE where its current intensity depends on the concentration of the chlorophenol present in solution (Fig. 4b). This could be assigned to a catalytic reaction of the phenol compound as a consequence of 2,6-dichlorophenol biocatalysis in presence of POX_{1B} enzyme and H_2O_2 as co-substrate. In the case of peroxidases such as HRP, the increase of reduction current was assigned to the generation of free phenoxy radicals as a consequence of the phenolic compound biocatalysis (Serra et al., 2001). In fact, peroxidases are enzymes which catalyze the oxidation of donor substrates by hydrogen peroxide in three steps (Munteanu et al., 1998). An oxidized enzyme intermediate (compound I) is formed in a first reaction (1) involving the reduction of hydrogen peroxide. Then, a two-step reduction of compound I generates radical products (Ph*) after oxidation of the donor substrate in each step (reactions (2) and (3). In reaction (2), a second compound is formed by the reduction of compound I in presence of the phenolic compound (Ph). In reaction (3), the POX_{1B} enzyme returns to its native form when compound II is reduced in presence of the phenolic compound (Ph) and $2H^+$ generating radical products (Ph*) and H_2O. When non-mediated electron transfer is used, reaction (4) is expected to occur. The cited reactions are summarized below:

$$POX_{1B} + H_2O_2 \rightarrow \text{CompoundI} + H_2O(k_1) \quad (1)$$

Table 1
Summary of the analytical parameters of the POX_{1B}-based biosensor for different phenolic compounds.

Phenolic compound	$-E_{red}$ (V/SCE)	Linear range (mol L^{-1})	Lower measured concentration (mol L^{-1})	Sensitivity (10^9 μA L mol^{-1})	Relative standard deviation ($n=5$)	$K_{m,app}$ (μmol L^{-1})
2,6-dichlorophenol	0.50	1×10^{-8} to 2×10^{-5}	1×10^{-8}	0.0015	1.5%	0.46
4-chlorophenol	0.46	1×10^{-12} to 2×10^{-5}	1×10^{-12}	1.9	5%	0.42
Pentachlorophenol	0.42	1×10^{-12} to 2×10^{-5}	1×10^{-12}	0.9	3.6%	0.11

$$\text{CompoundI} + \text{Ph} \rightarrow \text{CompoundII} + \text{Ph}*(k_2) \quad (2)$$

$$\text{CompoundII} + \text{Ph} + 2H^+ \rightarrow POX_{1B} + \text{Ph}* + H_2O(k_3) \quad (3)$$

$$\text{CompoundI} + 2e^- + 2H^+ \rightarrow \text{POD} + H_2O(k_s) \quad (4)$$

In the current research where POX_{1B} was used in enzyme catalysis and assuming that its biochemical characteristics are peroxidase like (El Ichi et al., 2008) as demonstrated above, the mechanism of the catalytic reaction should be the same as for the HRP. The reduction current observed at a negative potential of −0.4 V *vs*. SCE can be attributed to the reduction of a free radical phenoxy or to a highly oxidized enzyme intermediate formed during the reduction of substrate (compound I or II).

Linear increase of the current response was proportional to the added H_2O_2 concentrations in the range of 10 μM to 1 mM. The sensitivity of the Chitosan/POX_{1B}-based biosensor to H_2O_2 was already demonstrated in a previous work (El Ichi et al., 2009). In the present work, hydrogen peroxide is used as co-substrate to allow the detection of chlorophenols. In order to determine the minimal H_2O_2 concentration to add in the mixture for chlorophenols detection, two chrono amperometric assays were recorded in presence of 2×10^{-4} mol L^{-1} 2,6-dichlorophenol and successive additions of H_2O_2 from 1×10^{-5} to 1×10^{-3} mol L^{-1}. Negative control without 2,6-dichlorophenol in the mixture was made in the same conditions. A higher reduction current was observed in presence of 2,6-dichlorophenol in comparison to H_2O_2 amperometric response.

3.3. Sensitivity of the sensor

Sensitivity of the POX_{1B}-based biosensor was assayed using three reputed toxic chlorophenols: 2,6-dichlorophenol, 4-chlorophenol and pentachlorophenol. The chosen H_2O_2 concentration was 1×10^{-5} mol L^{-1} with regards to the chrono amperometric results. The POX_{1B}-based biosensor was able to detect 2,6-dichlorophenol, 4-chlorophenol and pentachlorophenol at very low concentrations (Fig. 5). These curves showed that the reduction current response was linear and proportional to the concentrations of the phenolic compounds in the mixture. In fact, 2,6-dichlorophenol can be detected in a linear range from 1×10^{-8} to 2×10^{-5} mol L^{-1}, when the 4-chlorophenol and pentachlorophenol are detected in the linear range from 1×10^{-12} to 2×10^{-5} mol L^{-1} (Table 1). The sensitivity of the biosensor was calculated from calibration curve obtained for the lower range of chlorophenol derivatives (Fig. 5). The sensitivity of the biosensor towards the 2,6-dichlorophenol, 4-chlorophenol and pentachlorophenol was respectively, 1.5×10^6, 1.9×10^9 and 0.9×10^9 μA L mol^{-1} (Table 1). These values are much higher than the reported ones in the case of the graphite-teflon-GOD-HRP composite electrodes since the sensitivity for the 4-chlorophenol and the pentachlorophenol was respectively, 4.1×10^4 and 2.4×10^3 μA L mol^{-1} (Serra et al., 2001). The values of the detection limits reported in Table 1 were concluded from the lower concentration of chlorophenol derivatives detected with significant measured signal of current. A detection limit of 1×10^{-8} mol L^{-1} was obtained in the case of 2,6-dichlorophenol and 1×10^{-12} mol L^{-1} in the case of 4-chlorophenol and pentachlorophenol.

$K_{m,app}$ values were calculated from calibration curves obtained with a concentration range of 1×10^{-7} to 1×10^{-6} mol L^{-1} of chlorophenol derivatives in presence of 1×10^{-5} mol L^{-1} hydrogen peroxide according to a Michaelis–Menten kinetic behaviour (Table 1). For comparison, the $K_{m,app}$ value for 4-chlorophenol (4.2×10^{-7} mol L^{-1}) and pentachlorophenol (1.1×10^{-7} mol L^{-1}) are much less than the obtained values for the same phenolic compounds using the graphite-teflon-HRP composites electrodes where the $K_{m,app}$ is 5.1×10^{-4} mol L^{-1} for 4-chlorophenol, and 4.0×10^{-3} mol L^{-1} for pentachlorophenol (Serra et al., 2001). Thus, the novel peroxidase POX_{1B} exhibits higher affinity towards phenolic compounds compared with HRP, tyrosinase and glucose oxidase. In addition, the POX_{1B}-based biosensor needed lower hydrogen peroxide concentrations. These results demonstrate a marked improvement compared to other detectors. In fact, the most sensitive tyrosinase biosensor, recently reported, has a detection limit of 2.5×10^{-8} mol L^{-1} for phenol (Wang et al., 2008). Detection limits obtained with composite multienzyme biosensors based on glucose oxidase-HRP and glucose oxidase-HRP-tyrosinase were respectively $(1.5\pm0.2)\times10^{-3}$ and $(1.3\pm0.1)\times10^{-3}$ mol L^{-1} (Serra

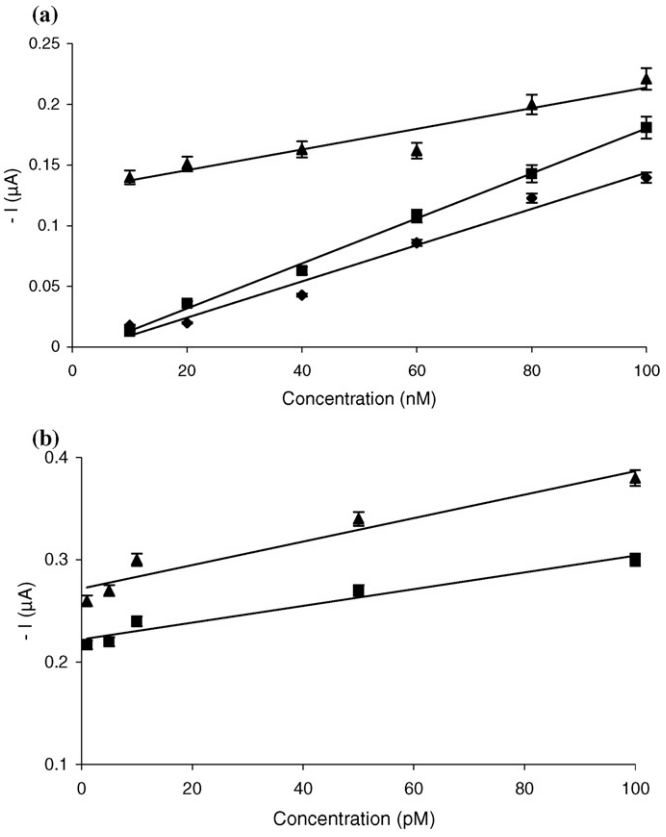

Fig. 5. Calibration curves obtained by measuring current at fixed potential of $E=-0.48$ V/SCE and in presence of 1×10^{-5} mol L^{-1} H_2O_2, (♦) 2,6-dichlorophenol; (■) 4-chlorophenol; (▲) pentachlorophenol, (a) concentration range of phenolic compounds from 1×10^{-6} to 1×10^{-5} mol L^{-1}; (b) concentration range of phenolic compounds from 1×10^{-11} to 1×10^{-10} mol L^{-1}.

et al., 2003). The difference in sensitivity could be assigned to the selectivity of the peroxidase for tested phenolic compounds.

These results demonstrate that the analytical performance of the POX_{1B}-based biosensor was better than reported for other enzyme-based biosensors for phenolic compounds detection. This improved performance could be related to the properties of the enzyme POX_{1B} and also the procedure of its immobilization into chitosan microspheres. A low cross-linker (glyoxal) concentration was used in the final step to allow the formation of the chitosan matrix on the gold electrode without denaturizing the enzyme. The optimization of chitosan particles preparation and enzyme attachment has given higher immobilization and activity yields compared to previous results (El Ichi et al., 2009).

The high porosity of chitosan polymer offers a more available immobilization area for the enzyme. At the same time, the spongy structure of chitosan microparticles facilitates the diffusion of substrates and electron transfer reactions. Other methods reported in the literature, such us incorporation within graphite-teflon matrices (Serra et al., 2001, 2003), covalent immobilization using mercaptopropionic acidic auto assembled monolayers (Imabayashi et al., 2001), derived sol–gel matrices (Kane et al., 1998; Rosatto et al., 2002) or silica-titanium gels (Rosatto et al., 1999) show lack of stability even if the sensitivity of the designed devices for pollutants monitoring was enhanced. In our case, the stability problem was resolved by the use of an optimized chitosan procedure cited above.

To test the effect of some interfering parameters such as temperature and pH, the POX_{1B}-based biosensor was tested in the presence of 2×10^{-4} mol L^{-1} 2,6-dichlorophenol and 5×10^{-5} mol L^{-1} H_2O_2 by varying temperature in the range of 15–40 °C and pH in the range of 5.8–7.4, as chitosan is soluble at pH ≤ 5.5 (Mello et al., 2006). A linear increase in the electrochemical response of the biosensor was noticed with increasing temperatures in the range 15–30 °C with regression factor of 0.99 before reaching a plateau at 40 °C. This can be explained by attaining conditions near the optimal temperature (30 °C) of POX_{1B} enzyme on the one hand, and speeding up of the catalysis reaction on the other. A similar result has been obtained using tyrosinase (Wang et al., 2008).

No effect of pH was observed on the electrochemical response of the biosensor. The POX_{1B}-based biosensor was operational at pH 7.4 as well as acidic pH. The efficiency of POX_{1B} enzyme at acidic pH has already been demonstrated in a previous work (El Ichi et al., 2008). The immobilization procedure using chitosan as a matrix and glyoxal as cross-linker enabled full activity and stability of the POX_{1B} enzyme to be retained.

3.4. Reproducibility and storage stability of the biosensor

Reproducibility of the current response and stability *vs*. storage of the POX_{1B}-based biosensor were investigated. All electrochemical assays achieved with 2,6-dichlorophenol, 4-chlorophenol and pentachlorophenol were performed in quintuplicate. The same POX_{1B}-based electrode was used for all assays to examine the repeatability of obtained results. The linearity of current response of the biosensor was reproducible for all tested concentration ranges and the electrochemical response of the biosensor was reproducible based on the low calculated relative standard deviation (RSD) values (Table 1).

Electrodes with different amounts of peroxidase POX_{1B} were also prepared to evaluate the effect of enzyme concentration on the electrochemical response of the biosensor. The measured reduction currents corresponding to electroactive enzyme concentrations of 4×10^{-9}, 1×10^{-9} and 8×10^{-10} mol cm^{-2}, were respectively −0.26, −0.16 and −0.06 μA, in presence of 5×10^{-5} mol L^{-1} H_2O_2, 1×10^{-6} mol L^{-1} 2,6-dichlorophenol. Thus, the electrochemical response of the biosensor varied proportionally with the immobilized enzyme amount.

In order to evaluate the long-term stability of the prepared biosensor, electrochemical response to 2×10^{-4} mol L^{-1} 2,6-dichlorophenol in presence of 5×10^{-5} mol L^{-1} H_2O_2 was assayed every 15 days during 3 months. The POX_{1B}-biosensor was stored in 10 mM PBS pH 7.4 at 4 °C when not in use. The biosensor retained the totality of its original response after the whole period with no need to regenerate the electrode surface. Such stability is very high compared to other biosensors, for example, a reported tyrosinase-based biosensor retained 53% of its original response after 2 months (Wang et al., 2008). The high stability of the POX_{1B} biosensor is due to both the properties of the enzyme and the immobilization procedure. In fact, stability of free POX_{1B} enzyme has already been demonstrated (El Ichi et al., 2008) with a loss of only 10% after 3 months storage. The immobilization of POX_{1B} on chitosan microspheres allows it to retain the total activity. Chitosan microspheres offer the immobilized enzyme a protective and biocompatible environment which prevents it from leaching during storage. This was also confirmed by measuring the electroactivity of the enzyme immobilized each month during storage. Assays are in course to evaluate the stability of the present biosensor for an extended period of time.

3.5. Rapidity

In addition to sensitivity, reproducibility and storage stability, the rapidity of the electrochemical response was the next parameter tested to evaluate the performance of the POX_{1B}-based biosensor in pollutants monitoring. Electrochemical measurements were recorded using chrono amperometry. A fast reduction current was obtained with time response less than 1 s. This current increased when higher concentrations of 2,6-dichlorophenol were added.

4. Conclusion

The peroxidase POX_{1B} biosensor was able to detect very low concentrations of pollutants since detection limits attained 1×10^{-8} mol L^{-1} for 2,6-dichlorophenol and 1×10^{-12} mol L^{-1} for 4-chlorophenol and pentachlorophenol. The performance of the biosensor evaluated with regards to sensitivity, stability, reproducibility and rapidity has been demonstrated to be superior to other biosensors described in the literature. The immobilization procedure using chitosan as a matrix allowed not only high enzyme loading onto the electrode surface with respect to its native conformation but also easy electron transfer reactions thanks to its porosity. In addition, the method was simple with no co-enzyme and no extra mediator needed. The novel POX_{1B}-based biosensor is multifunctional and reusable with a low cost. Furthermore, the response time of the biosensor, which was less than 1 s permits a shorter time of analysis. This is particularly advantageous in applications requiring a large number of samples to be monitored in situ and in real time. The detection of phenolic compounds, especially pentachlorophenol, in wastewater samples requires an accurate and reliable analytical tool since these toxic substances are of great health concern. The present biosensor can also be applied for monitoring other environmental pollutants. Some modifications of the immobilization procedure using chitosan microparticles are being tested to make possible the use of a POX_{1B}-based biosensor in more acidic pH.

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