Full Paper

An Amperometric Biosensor for *trans*-Resveratrol Determination in Aqueous Solutions by Means of Carbon Paste Electrodes Modified with Peroxidase Basic Isoenzymes from Brassica Napus

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Abstract
The catalytic properties of peroxidase basic isoenzymes (PBI's) from *Brassica napus* towards *trans*-resveratrol (t-Res) oxidation were demonstrated by the first time by conventional UV-visible spectroscopic measurements. The enzymatic reaction rate was studied under different experimental conditions and the kinetics parameters were determined. An amperometric biosensor based on *Brassica napus* PBI's to determine t-Res is also proposed by the first time. The method employs a dialysis membrane covered, PBI's entrapped and ferrocene (Fc)-embedded carbon paste electrode (PBI's-Fc-CP) and is based on the fact that the decreased amount of H_2O_2 produced by the action of PBI's is proportional to the oxidised amount of t-Res in the solution. Comparative amperometric experiments showed that, in spite of PBI's activity was much lower than commercial horseradish peroxidase (HRP) activity, t-Res was a much better substrate for PBI's biosensors than those biosensors constructed by using HRP. The PBI's-Fc-CP biosensors showed a very good stability during at least twenty days. The reproducibility and the repeatability were 4.5% and 8.3%, respectively, showing a good biosensor performance. The calibration curve was linear in the t-Res concentration ($c_{t\text{-Res}}$) range from 1×10^{-6} to 2.5×10^{-5} M, with a sensibility of $(2.31 \pm 0.05) \times 10^6$ nA M^{-1}. The lowest $c_{t\text{-Res}}$ value measured experimentally for a signal to noise ratio of 3:1 was 0.83 μM.

Keywords: t-Resveratrol, Amperometric biosensors, Peroxidase basic isoenzymes, *Brassica napus*, Enzymatic kinetic parameters

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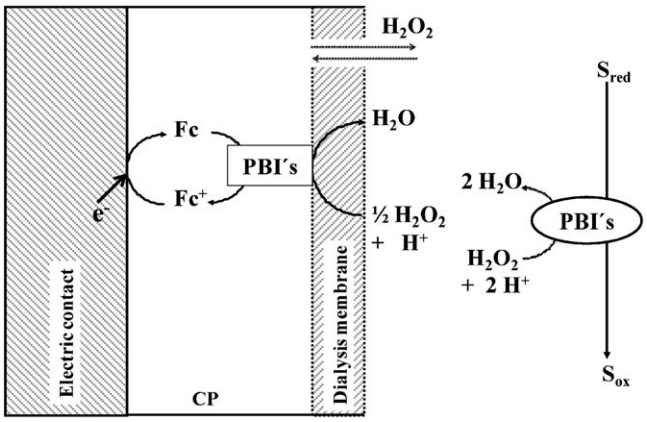
1. Introduction

It is well known that the phenolic compound *trans*-resveratrol or 3,5,4′-trihydroxystilbene (t-Res) is a naturally occurring phytoalexin, a class of antiobiotic produced as a part of a plant defense system against diseases. It came to scientific attention as an antioxidant [1, 2], an anticancer agent [3] and a phytoestrogen [4]. Although present in other plants, such as eucalyptus, spruce, and lily, and in other foods such as mulberries and peanuts, the most abundant natural sources of t-Res are *Vitis vinifera*, labrusca and muscadine grapes, which are used to make wines. It occurs in the vines, roots, seeds and stalks, but its highest concentration is in the skin, which contains 50 – 100 μg/g [5].

The usually proposed methods for the determination of t-Res are based on HPLC-UV and HPLC-fluorescence detection [6 – 8]. The application of liquid chromatography-electrospray tandem mass spectroscopy has been also studied [9, 10]. The use of capillary electrophoresis with electrochemical detection has been also described [11].

The t-Res electrochemical behavior has been previously studied in our laboratory by cyclic voltammetry (CV) in acetonitrile (ACN) + 0.1 M $NaClO_4$ as the reaction medium [12]. An oxidation peak at about 1 V was observed at platinum, gold and glassy carbon electrodes, with a shoulder at about 1.05 V vs. the saturated calomel electrode (SCE). No complementary cathodic peak was found when the potential scan rate direction was reversed, putting clearly in evidence a complicated electrochemical reaction mechanism. Besides, when a second consecutive scan was performed on the same working electrode, the electrochemical signal was lost as a consequence of the fouling of the electrode surface by the deposition of possible non-electroactive polymers generated by the t-Res oxidation products. Recently, the voltammetric behavior of t-Res on carbon paste (CP) electrodes by using CV and square wave voltammetry has been investigated in 1.0 mM KCl + 0.1 M HNO_3 as supporting electrolyte [13]. Results reported showed a good agreement with those previously found by us [12]. This complicated electrochemical behavior has been also found when a phenolic species is the responsible of the



Scheme 1. Two-stage peroxidase reaction scheme for detecting phenolic compounds using a membrane covered PBI's-Fc-CP biosensor. S_{red} and S_{ox} are the reduced and the oxidized forms of the phenolic compound, respectively.

compound electrochemical activity [14, 15]. This fact makes difficult the application of usual electroanalytical techniques to determine the t-Res natural antioxidant directly on conventional electrodes.

In this article, we propose a peroxidase basic isoenzymes (PBI's)-based amperometric biosensor to determine t-Res in solutions prepared with the commercial reagent. PBI's are extracted from *Brassica napus* obtained from local markets. The method employs a membrane-covered, PBI's – entrapped, and ferrocene (Fc)-embedded carbon paste electrode (PBI's-Fc-CP). This methodology is based on the fact that phenolic compounds can work as electron-donors for peroxidases in the catalytic reduction of H_2O_2 [16, 17]. This approach allows detecting the decrease in H_2O_2 concentration in a solution after the oxidation of t-Res by the PBI's in the presence of H_2O_2; PBI's acts in cascade in solution and at the electrode surface. The separation of the electrode surface from the solution by a semipermeable membrane minimizes the electrical noises as well as the fouling of the electrode surface by the polymerized products of oxidized t-Res, which may interfere with electrochemical measurements. The scheme for the detection of t-Res based on the two-stage peroxidase reactions using the PBI's-Fc-CP electrode is shown in Scheme 1.

The performance of these biosensors was compared with that obtained for similar biosensors, using HRP (HRP-Fc-CP) instead of PBI's.

2. Experimental

2.1. Plant Material

Freshly collected turnip roots were purchased from the local market and frozen at $-20\,^{\circ}C$ until were used.

2.2. Chemicals

Hydrogen peroxide (30% v/v), pH 7.00 phosphate buffer solutions (PBS), sodium acetate, acetic acid, NaCl, HCl and NaOH were Merck p.a.; t-Res, Fc, *o*-dianisidine, tris-hydroxymethyl amino methano (Tris) and HRP Type VI peroxidase were purchased from Sigma and used as received. All solutions were prepared using water purified by a Labconco WaterPro Mobile System, Model 90901-01 (HPLC grade water). The concentration of H_2O_2 was determined spectrophotometrically at $\lambda_{max} = 240$ nm ($\varepsilon = 43.6\ M^{-1}\ cm^{-1}$) [18]. ACN and methanol were Sintorgan (HPLC-grade). Stock solutions of t-Res were prepared in ACN and were kept in the dark at a temperature below $0\,^{\circ}C$. Aliquots were then added daily to the corresponding reaction media. Solutions of different pH values were obtained from pH 7.00 PBS by the addition of various volumes of 1 M HCl or NaOH.

2.3. Total Enzyme Extraction and Peroxidase Activity Determination

Roots were homogenized in a mortar with 10 mM pH 4.00 sodium acetate/acetic acid buffer, containing 1 M NaCl (1 g fresh roots weight per 2 mL of buffer) at $4\,^{\circ}C$. Homogenates were centrifuged at 5000 rpm for 5 min. The supernatants were considered as total peroxidase extracts (TPE). They were used in order to purify peroxidase isoenzymes and to determine total peroxidase activity. It was determined with *o*-dianisidine as substrate [19] and expressed in international unit (IU), which was defined as the amount of enzyme forming 1 μmol of product in 1 min under the experimental conditions employed.

2.4. Enzyme Purification

Purification of peroxidase isoenzymes (PI's) was performed by ion – exchange chromatography on DEAE Sephacel (SIGMA) columns. Samples of 2 mL of TPE, previously dialyzed, were loaded on a DEAE Sephacel column (2×32 cm) equilibrated with a pH 8.60 Tris/HCl buffer. The column was washed with 100 mL of this buffer, and bound proteins were eluted using 200 mL of 0.3 M NaCl in the same buffer at a flow rate of 1 mL min^{-1} controlled with a peristaltic pump (BIO RAD). Fractions (5 mL each) were collected and monitored to determine their peroxidase activity by their A_{403nm} [19]. Fractions containing the major cationic peroxidases were mixed and lyophilized before they were used.

2.5. Analysis of Isoenzyme Patterns

Peroxidases were identified by isoelectric focusing (IEF) in a pH range of 3.0 – 10.0 on polyacrylamide gels (SIGMA) following the procedure previously described by González

et al. [19]. A Bio-Rad's IEF standard of proteins was used. Gels were stained with benzidine (SIGMA) and H_2O_2 to detect peroxidase activity and with Coomasie Brilliant Blue R-250 to detect protein markers p*I*, following a procedure previously described [19–22].

2.6. Apparatus and Experimental Measurements

Cyclic voltammograms and amperometric measurements were performed with an EG&G PARC Model 273 potentiostat controlled by PAR270 electrochemical analysis software. The scan rate was 0.050 V s^{-1}. Electrochemical measurements were carried out in a 5 mL Pyrex cell. The working electrode was a carbon paste (CP) disk of 1.6 mm diameter obtained from BAS Chemical Company. The counter electrode was a platinum foil of large area (≈ 2 cm^2). An aqueous SCE was used as reference electrode. Amperometric measurements were performed at a potential of 0 V vs. SCE in solutions stirred at 1600 rpm (see below).

The CP was BAS (CF-100). Fc was used as a redox mediator. Biosensors were constructed by using the following procedure: given amounts of PBI's, Fc and CP were mixed in a weight ratio of 3.33:1:15 and used to fill up CP electrodes. The electrode surface was then covered with a dialysis membrane (Spectrum Co, Houston, TX, cut-off molecular weight 100), which was fixed at the electrode side part with a Teflon laboratory film and an O-ring. They were stored at 4 °C in pH 7.00 PBS when they were not in use. Those biosensors prepared with HRP were obtained in a similar way as that previously described for PBI's biosensors, but the weight ratio of HRP, Fc and carbon paste was 1:1:15, respectively. The different ratio between peroxidases and Fc and CP amounts used to construct PBI's and HRP biosensors were chosen to obtain amperometric current responses in the same order of magnitude for both biosensors. At this point, it is important to remark that PBI's show an activity quite lower than the activity of HRP, i.e., 981 736 IU/mg of solid (HRP) versus 4030 IU/mg solid (PBI's), measured as indicated is Section 2.3. This difference in activity could be explained by the fact that commercial HRP type VI used in this work contains several peroxidase isoenzymes as it is shown in Figure 1, in agreement with results previously reported [23]. Sigma type VI HRP showed about three peroxidase bands, i.e., basic and acidic isoenzymes (Line HRP in Fig. 1) versus the only one for PBI's (Line PBI's in Fig. 1). However, PBI's biosensors made as described above showed amperometric currents about two times higher than those obtained with HRP biosensors under the same experimental conditions (see Sec. 3.3.2), in spite of the enzymatic activity, expressed as IU, was about 150 times higher for HRP than for PBI's.

Experiments performed with and without bubbling pure nitrogen in solutions did not show any significant difference. Therefore, measurements were carried out in non-deoxygenated solutions. UV-visible spectra were recorded immediately after the preparation of solutions by using a Hewlett-Packard Model 8452A spectrophotometer equipped with a temperature controller. Silica cell was of 1 cm pathlength. Measurements of pH were performed with an Orion Model 720A pH-meter, which was calibrated daily with three commercial buffers. Experiments were performed at 25.0 ± 0.2 °C.

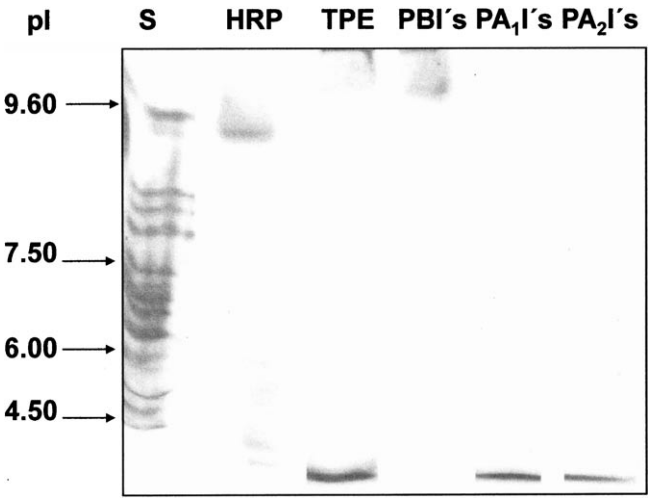


Fig. 1. IEF of TPE and partially purified isoenzymes in the pH range from 3.00 to 10.00. Line S: standard of p*I*. Line HRP: commercial HRP Type VI purchased from Sigma. Line TPE: total peroxidase extracts. Line PBI's: peroxidase basic isoenzymes. Lines PA_1I's and PA_2I's: peroxidase acidic isoenzymes.

3. Results and Discussion

3.1. Peroxidase Isoenzymes

Simple extraction and purification methods were applied in order to obtain TPE and several peroxidase isoenzymes with p*I* values from 9.6 to 3.6, which were detected by IEF (Fig. 1). A main group of peroxidase basic isoenzymes (PBI's $pI \geq 9.60$), and a group of peroxidase acidic isoenzymes (PAI's) were purified. PBI's were then selected to perform the following experiments.

3.2. Spectroscopic Measurements

The catalytic properties of PBI's towards t-Res oxidation were studied by conventional UV-visible spectroscopic measurements, using pH 7.00 PBS as the reaction medium, where t-Res presents an absorption band with two shoulders at $\lambda_{max} = 307$ nm ($\varepsilon = 3.12 \times 10^4$ M^{-1} cm^{-1}) and at $\lambda_{max} =$ 317 nm ($\varepsilon = 3.08 \times 10^4$ M^{-1} cm^{-1}). The enzymatic reaction rate was studied following the decrease in the shoulder at $\lambda = 317$ nm with the time, employing the initial rates (v_i) method. The effect of t-Res ($c_{t\text{-}Res}$), H_2O_2 (c_{H2O2}) and PBI's ($c_{PBI's}$) concentrations on enzymatic reaction was analyzed. The optimum concentration values of H_2O_2 and PBI's were 2 mM and 10 nM, respectively. A decrease in v_i was observed at a c_{H2O2} higher than about 2.5 mM, in good

Table 1. Constant kinetics for H_2O_2 at different t-Res concentrations as well as for t-Res at different H_2O_2 concentrations obtained during their oxidation by basic isoenzymes from *Brassica napus*. $c_{PBI's} = 10$ nM

$c_{t\text{-}Res}$ (μM)	V_{max} (nM s^{-1})	K_A (μM)
31.5	30.1 ± 1.5 [a] (32.5 ± 0.6) [b]	1.0 ± 0.1 [a] (1.2 ± 0.2) [b]
21.0	24 ± 1 [a] (23.4 ± 0.4) [b]	0.7 ± 0.1[a] (0.5 ± 0.1) [b]
15.4	22 ± 1 [a] (18.9 ± 0.2) [b]	0.52 ± 0.05 [a] (0.2 ± 0.4) [b]
	$V_{max} = 2\ c_{PBI's}\ k_3\ c_{t\text{-}Res}$ $k_1 = (2.2 \pm 0.3)$ [a] $\times 10^6$ M^{-1} s^{-1}	$K_A = (k_3/k_1)\ c_{t\text{-}Res}$ $k_1 = (1.5 \pm 0.6)$ [b] $\times 10^6$ M^{-1} s^{-1}
c_{H2O2} (mM)	V'_{max} (nM s^{-1})	K_B (μM)
1.50	17528 [c] (117.2 ± 0.1) [d]	100 ± 18 [c] (58.3 ± 0.1) [d]
0.51	92 ± 12 [c] (72.2 ± 0.2) [d]	44 ± 8 [c] (30.5 ± 0.1) [d]
0.10	48 ± 6 [c] (24.8 ± 0.2) [d]	20 ± 3 [c] (6.4 ± 0.1) [d]
	$V'_{max} = 2\ c_{PBI's}\ k_1\ c_{H2O2}$ $k_3 = (1.9 \pm 0.1)$ [c] $\times 10^5$ M^{-1} s^{-1}	$K_B = (k_1/k_3)\ c_{H2O2}$ $k_3 = (2.1 \pm 0.3)$ [d] $\times 10^5$ M^{-1} s^{-1}

[a] and [c]: Parameters calculated from v_i^{-1} vs. c^{-1} plots (Lineweaver-Burk plots).
[b] and [d]: Parameters calculated from the best fitting of v_i vs. c experimental plots by using the theoretical equations which describe the system (Eqns. 4 and 5).

agreement with previous reports which indicate that high c_{H2O2} inhibit peroxidase enzymatic reactions [18].

Steady-state kinetics peroxidase reactions has been described as a ping-pong scheme with compound I and compound II formation [24]. The steady-state rate equation for these two-substrate two product reactions is well known [24–26]. Therefore, the dependence of v with H_2O_2 as well as t-Res can be expressed as indicated in Equations 1 and 2.

$$v = V_{max}\ c_{H2O2}\ (K_A + c_{H2O2})^{-1} \quad (1)$$

$$v = V'_{max}\ c_{t\text{–}Res}\ (K_B + c_{t\text{–}Res})^{-1} \quad (2)$$

Definitions of V_{max}, K_A, V'_{max} and K_B are given in Table 1, where k_1 and k_3 are rate constants for the reaction of the enzyme with H_2O_2 and compound II with t-Res, respectively. Both, plots of v_i^{-1} vs. c^{-1} (Lineweaver–Burk plots) [24] and the fitting of v_i vs. c experimental plots by using the theoretical equations which describe the system (Eqs. 1 and 2) were used to calculate the apparent constants (K_A and K_B) and the maximum reaction rates (V_{max} and V'_{max}) under the different experimental conditions. Kinetics constants for H_2O_2 and t-Res were strongly dependent on t-Res and H_2O_2 concentrations, respectively (Table 1). Values obtained for parameters by different methods agree satisfactorily. Besides, they are in good agreement with those reported in the literature for related substrates [25, 26].

3.3. Electrochemical Measurements

3.3.1. Responses of PBI's-Fc-CP Biosensors Towards H_2O_2

A cyclic voltammogram recorded in unstirred pH 7.00 PBS for the PBI's-Fc-CP biosensor showed an increase in the anodic current at potentials higher than 0.2 V vs. SCE, which corresponds to the oxidation of Fc to Fc^+. A cathodic peak with a peak potential at about 0.2 V was observed when the potential sweep direction was reversed at 0.5 V, which can be assigned to the reduction of Fc^+ to Fc at the electrode surface. On the other hand, a cyclic voltammogram recorded when H_2O_2 was added to the reaction medium showed a cathodic current at potentials more negative than -0.3 V vs. SCE, which corresponds to the reduction of H_2O_2 at the biosensor surface. Therefore, the base current was practically zero between -0.3 and 0.2 V at pH 7.00. This pH value was chosen because of it was the optimum pH for the enzymatic reaction (see below, Sec. 3.3.3).

When only H_2O_2 was added to the stirred reaction medium, the enzymatic reaction took place between PBI's on the electrode surface and H_2O_2 penetrated into the inner layer between the semipermeable membrane and the electrode surface. H_2O_2 was reduced to H_2O by PBI's and the enzyme was reduced to its native form by Fc, which was oxidized to Fc^+. The Fc^+ was then immediately reduced to Fc at the electrode hold at a potential of 0 V vs. SCE. The steady-state reduction currents (I_{ss}) obtained after the addition of H_2O_2 different aliquots are shown in Figure 2a. The differences between the initial base current and I_{ss}'s (ΔI_{ss}) were proportional to the H_2O_2 bulk concentration, showing a Michaelis–Menten type saturation (see Fig. 2b).

Plots of ΔI_{ss}^{-1} vs. c^{-1}_{H2O2} were linear. Average values of $I_{max,H2O2}$ and $K_{A,H2O2}$ of (2.36 ± 0.08) μA and (0.76 ± 0.06) mM, respectively, were calculated from the intercept and the slope of those plots from five replicated measurements. Amperometric experiments similar to those previously described for PBI's-Fc-CP biosensors were performed using HRP in replaced of PBI's. Values of $I_{max,H2O2}$ and $K_{A,H2O2}$ determined for the HRP-Fc-CP biosensor were (2.2 ± 0.4) μA and (1.0 ± 0.2) mM, respectively. These results suggest that H_2O_2 is a better substrate for PBI's than for HRP. In addition, PBI's biosensors showed a better resistance to the inhibition by H_2O_2 than those biosensors constructed with HRP. Therefore, ΔI_{ss} values decrease for $c_{H2O2} \geq 1.5$ mM (results not shown) for HRP biosensors, putting clearly in evidence an inhibition of the enzymatic reaction, while this

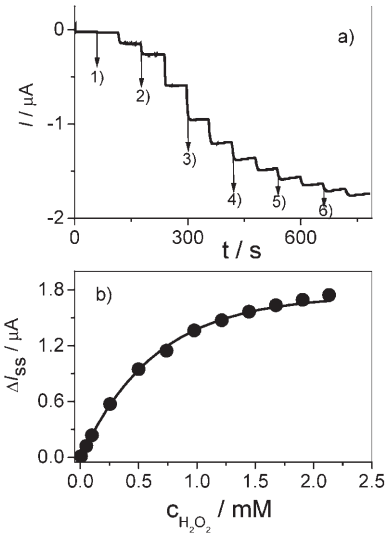


Fig. 2. a) Steady-state current responses on the addition of different H_2O_2 concentrations at the stirred pH 7.00 PBS reaction medium measured with a dialysis membrane covered PBI's-Fc-CP biosensor. c_{H2O2}: 1) 0.005; 2) 0.256; 3) 0.739; 4) 1.21; 5) 1.68, and 6) 2.13 mM. b) Differences between the base and the steady-state currents, ΔI_{ss}, as a function of c_{H2O2} under the same experimental conditions as (a).

behavior was not found with PBI's biosensors, at least at c_{H2O2} up to 2.2 mM as it can be observed in Figure 2b.

3.3.2. *Application of PBI's-Fc-CP Biosensors to the Determination of t-Res*

When both, PBI's and t-Res, were added to the stirred reaction medium composed by pH 7.00 PBS and a given H_2O_2 bulk concentration, the enzymatic catalytic cycle took place in the solution bulk. The oxidized PBI's, produced as a consequence of its reaction with H_2O_2, are reduced back to its native state by t-Res. The decrease of H_2O_2 in the solution bulk was detected as a decrease in the Fc^+ reduction current at the biosensor surface (see Scheme 1).

Thus, the addition of H_2O_2 to the pH 7.00 PBS + 50 nM PBI's reaction medium produced a steady limiting current ($I_{s,lim}$) at 0 V vs. SCE after 1 min (see Fig. 3a), which corresponds to the reduction of Fc^+ generated by the PBI's catalyzed reduction of H_2O_2 to H_2O. The $I_{s,lim}$ was linear with c_{H2O2} up to about 200 μM and then was leveled off, indicating a H_2O_2-saturated condition. The difference between $I_{s,lim}$ with and without t-Res ($\Delta I_{s,lim}$) corresponds to the c_{H2O2} decrease as a consequence that the enzymatic reaction was taking place in the bulk solution. $\Delta I_{s,lim}$ values obtained after the addition of different aliquots of t-Res to the solution composed by pH 7.00 PBS + 50 nM PBI's in the presence of different H_2O_2 bulk concentrations are shown in Figure 3b, where a typical saturation shape can be observed.

Similar studies to those previously described were also performed for HRP-Fc-CP biosensors. However, the $I_{s,lim}$ was linear with c_{H2O2} up to about 80 μM for HRP and, then, it was leveled off. Plots of $\Delta I_{s,lim}^{-1}$ vs. $c_{t\text{-}Res}^{-1}$ were linear for

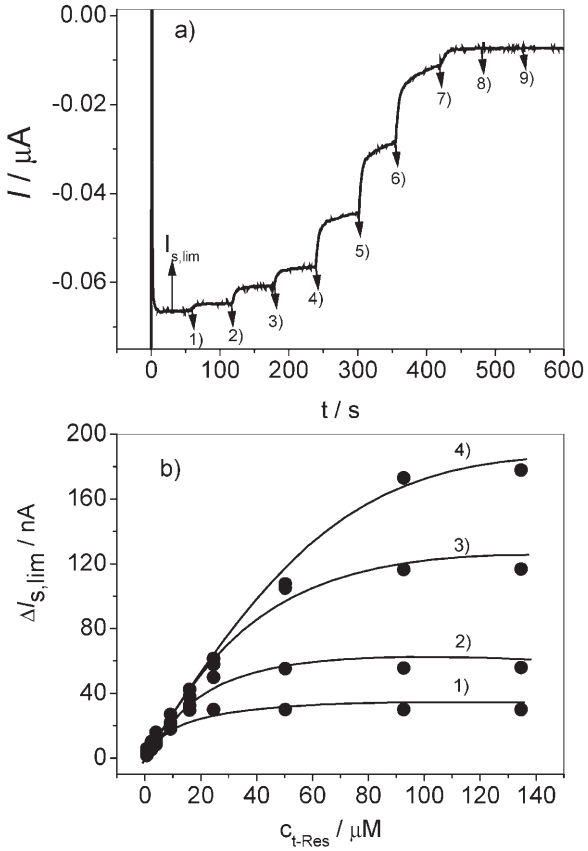


Fig. 3. a) Steady limiting current ($I_{s,lim}$) response obtained by the addition of 30 μM H_2O_2 and after the stepwise addition of t-Res detected with a dialysis membrane covered PBI's-Fc-CP biosensor at 0 V vs. SCE in pH 7.00 PBS + 50 nM PBI's under stirring. $c_{t\text{-}Res}$: 1) 0.70; 2) 2.37; 3) 4.04; 4) 9.23; 5) 16.10; 6) 24.60; 7) 50.27; 8) 92.67, and 9) 134.66 μM. b) Differences between steady limiting currents with and without t-Res in the reaction medium, $\Delta I_{s,lim}$, as a function of t-Res concentration recorded at different H_2O_2 bulk concentration. c_{H2O2} = 1) 15; 2) 30; 3) 60, and 4) 100 μM.

both, PBI's-Fc-CP and HRP-Fc-CP biosensors. Values of $I_{max,\,t\text{–}Res}$ and $K_{B,t\text{–}Res}$ (for PBI's-Fc-CP biosensors) and $I'_{max,\,t\text{–}Res}$ and $K'_{B,t\text{–}Res}$ (for HRP-Fc-CP biosensors) were obtained from those plots at different c_{H2O2}. They are shown in Figure 4. As can be observed, plots of $I_{max,\,t\text{–}Res}$ and $I'_{max,\,t\text{–}Res}$ vs. c_{H2O2} indicate no significant difference between maximum currents for PBI's and HRP biosensors (Fig. 4a). However, values of $K_{B,t\text{–}Res}$ and $K'_{B,t\text{–}Res}$ vs. c_{H2O2}showed that $K_{B,t\text{–}Res}$ are significantly smaller than those for $K'_{B,t\text{–}Res}$ for a given c_{H2O2} (Fig. 4b). These results along with the fact that a much lower activity of PBI's than HRP is enough to get similar amperometric responses (see Sec. 2.6) put clearly in evidence that t-Res is a much better substrate for PBI's biosensors than HRP biosensors, which it would be a challenge to develop PBI's biosensors to determine t-Res in real samples.

3.3.3. *Biosensor Responses at Different pH Values*

The effect of the pH of the buffer solution on response of the PBI's-Fc-CP biosensors towards t-Res was investigated

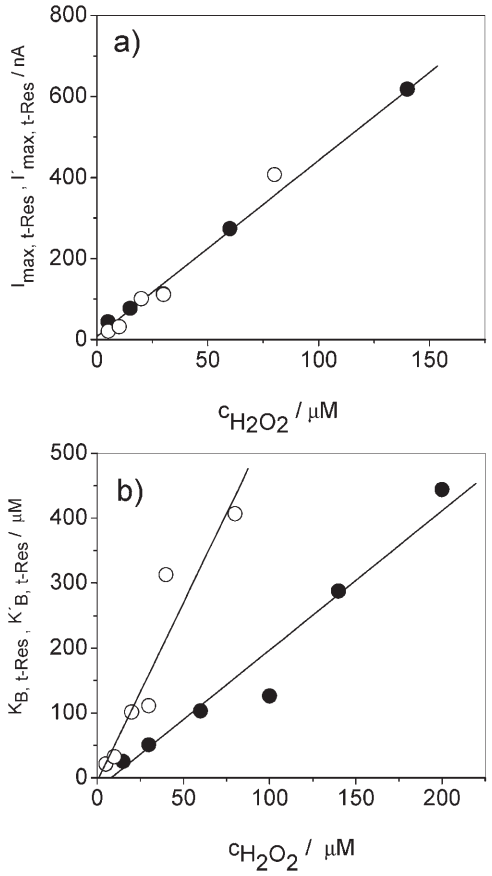


Fig. 4. Dependence of t-Res kinetic parameters with c_{H2O2} during its oxidation with: (●) PBI's-Fc-CP and (○) HRP-Fc-CP biosensors.

between pH 5.0 and pH 9.0. The $I_{max,\ t\text{–}Res}$ values obtained after the addition of different $c_{t\text{-}Res}$ in the presence of 100 μM H_2O_2 and 50 nM PBI's increased from pH 5.0 to 7.0. Then, $I_{max,\ t\text{–}Res}$ values decreased at higher pH's. Therefore, studies were performed at an optimum pH value of 7.00, which was also the optimum pH value for HRP-Fc-CP biosensors.

3.3.4. Biosensor Statistical Parameters

The PBI's-Fc-CP biosensor reproducibility was tested by measuring $\Delta I_{s,\ lim}$ values of five different bioelectrodes for a $c_{t\text{-}Res}$ of 9.35 μM in a solution of pH 7.00 PBS + 50 nM PBI's + 100 μM H_2O_2 as the reaction media. A percent relative standard deviation (%*RSD*) of 4.5% was obtained.

The repeatability assays were performed making six consecutive amperometric measurements with the same biosensor. %*RSD* for $c_{t\text{-}Res}$ of 9.35 and 19.64 μM were 8.3% and 6.8%, respectively, when a PBI's-Fc-CP biosensor was used in pH 7.00 PBS + 50 nM PBI's in the presence of 100 μM H_2O_2.

The stability of PBI's-Fc-CP biosensors was tested by using the same biosensor to measure the current responses obtained after the addition of different $c_{t\text{-}Res}$ to the previously reaction medium indicated. The current responses were practically constants between the experimental errors until about twenty days, showing a good stability of PBI's in biosensors. A decrease of 40% in current responses was observed after the fifth week. The stability found in PBI's biosensors studied in this work is in good agreement with results previously reported regarding an increase in stability of immobilized peroxidase on solid surfaces [27], as compared with peroxidase in solution, where it becomes inactivated during its reaction with phenolic compounds [28]. Therefore, enzyme immobilization is an excellent technique due to its high storage capability and better control of the catalytic process [27].

A linear relationship between $\Delta I_{s,\ lim}$ vs. $c_{t\text{-}Res}$ was obtained in the range from 1×10^{-6} to 2.35×10^{-5} M. The calibration curve can be expressed by a least square procedure as (eight experimental points were taken into account):

$$\Delta I_{s,\ lim} = (2.31 \pm 0.05) \times 10^6 \text{ nA M}^{-1}\ c_{t\text{-}Res} + (5.1 \pm 0.6) \quad (r = 0.9992) \tag{3}$$

where $\Delta I_{s,\ lim}$ is expressed in nA and $c_{t\text{-}Res}$ in M. The lowest $c_{t\text{-}Res}$ value measured experimentally for PBI's-Fc-CP biosensors for a signal to noise ratio of 3:1 was 0.83 μM (0.19 mg L^{-1}). This value is compared reasonably well with those reported for related polyphenolic compounds determined with other biosensors [29, 30]. In spite of it is three times higher than that value calculated for t-Res by capillary electrophoresis with electrochemical detection [11] and about twenty times higher than the value determined for t-Res by liquid chromatography-electrospray tandem mass spectroscopy [10], the simplicity in their construction as well as the low cost of the equipment required makes this methodology very convenient as a screening rapid method for the determination of t-Res in most real food matrixes (wines, teas, etc.) which contain t-Res amounts quite above the detection limits indicated. However, it should be indicated that PBI's-Fc-CP biosensors would not be able to discriminate t-Res from other polyphenolic compounds, which are usually present in natural extracts and could be very probably substrates of PBI's. Therefore, results presented and discussed in this work are preliminary ones, where t-Res was chosen as a model polyphenolic compound to prove the catalytic activity of PBI's. Nowadays, new experiments are being undertaken in our Laboratory for applying this electroanalytical methodology to determine the total content of polyphenolic compounds in real matrixes, particularly, in Argentinean wine samples obtained from different regions, where the wine industry plays an important role in the national economy.

4. Conclusions

Results found demonstrate, for the first time, that t-Res is a substrate of *Brassica napus* peroxidase basic isoenzymes (PBI's). The reactivity of these PBI's with H_2O_2 ($k_1 \approx 2 \times 10^6$ M^{-1} s^{-1}) and with t-Res ($k_3 \approx 2 \times 10^5$ M^{-1} s^{-1}) suggests that *Brassica napus* PBI's reacts with H_2O_2 in a similar way that other peroxidases, while t-Res appears to be a good

substrate for intermediate I and II compounds reduction. On the other hand, covered dialysis membrane PBI's-Fc-CP biosensors allowed performing the t-Res detection and quantification in a potential range where noises and background currents were low and the contribution to the response from interfering compounds in real samples may be little. The absence of the fouling phenomenon of the electrode surface for t-Res oxidation products could be also achieved. It has also been found that H_2O_2 is a better substrate for PBI's than for HRP. In addition, PBI's biosensor showed a better resistance to the inhibition by H_2O_2 than those constructed by HRP.

Moreover, this analytical methodology has some advantages over the classic HPLC methods, such as: the electrochemical instrumentation is cheaper, a lesser amount of solvents is required and the analysis time can be notably shortened. These advantages would make the use of PBI's biosensor as a useful tool for a rapid screening in the determination of antioxidants in food matrixes.

On the other hand, the biosensor stability, the reproducibility, the repeatability and the detection limit showed a good performance for PBI's biosensors towards the t-Res natural antioxidant.

Another advantage of this biosensor is that its response is unaffected by oxygen dissolved in samples. Therefore, it is possible to use this methodology to monitor the level of the total phenols in wine during the course of winemaking as well as in other samples, such as tea, in real time during food processing.

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