Romanian Biotechnological Letters *Vol. 17, No.5, 2012*

ORIGINAL PAPER

ELECTROCHEMICAL STUDY OF POLYPHENOLS WITH AMPEROMETRIC TYROSINASE BASED BIOSENSORS

Received for publication, February 01, 2012
Accepted, August 25, 2012

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Abstract

This paper describes the preparation of a novel biosensor based on tyrosinase immobilized onto single wall carbon nanotubes screen-printed electrodes modified with iron(II) phthalocyanine, and its application for measuring the polyphenols compounds in vegetables samples. The tyrosinase preserves its biocatalytic activity within the carbonaceous matrix. A noticeably defined reduction peak proportional to the catechin concentration was observed in cyclic voltammetry, which correspond to the reduction of enzymatically produced quinone at the electrode surface. Different analytical parameters influencing the biosensor performance have been studied such as applied potential and pH. In the optimal conditions, the biosensor presented a linear response range for catechin from 5 to 250□M, applying a potential of 0.15V versus Ag/AgCl, with a sensitivity of 0.094 □A □M^{-1} and a detection limit of 0.89□M. The biosensor was used in the polyphenols determination in green tea extracts and the results were compared with the Folin–Ciocalteau method and the biosensor demonstrated suitability to the quantification of the total polyphenols.

Key words: biosensor, tyrosinase, phthalocyanine, polyphenol, green tea

Introduction

The polyphenols are widespread distributed in the plants, mainly in the form of bioproducts produced from plant metabolism and they are found in foods of vegetable origin [1]. They are generally divided in three groups: phenolic acids, flavonoids and tannins [2]. Recently, they have attracted much attention due to their health benefits [3-4]. Polyphenolic compounds are responsible for the antioxidant capacity in most plant-derived products. This compounds act as free radical scavengers, metal chelating agents, inhibition of lipoprotein oxidation and also these compound exhibit properties related to the prevention of oxidation, inflammation, allergies and cancer [5-8]. Moreover, polyphenols influence in the quality of vegetables as color, aroma, texture and organoleptic attributes of beverages as wine, olive oil, coffee and tea [9].
Numerous analytical methodologies have been proposed in order to quantify phenolic compounds in food, beverages, pharmaceutics and biological fluids. These techniques include gas and liquid chromatography and spectroscopic techniques [10-12]. In spite of the existence of all them, the Folin–Ciocalteau method is the most used [13] and even though this method is well established for this purpose, simpler and faster methods are required for quality-control purposes during or after the food processing.
Electrochemical sensors and biosensors can be a promising alternative to these techniques. Electroanalytic technique based on biosensors is an interesting method due to simplicity, low cost, high sensitivity and possibility of miniaturization [14]. Enzymes are complex proteins

that produce a specific chemical reaction in other substances without themselves being modified carrying out as biocatalysts by lowering the activation energy [15]. For the detection of phenolic compounds, biosensors based on tyrosinase have been developed [16-18]. Tyrosinase catalyzes the transformation of monophenols to diphenols and also the reaction of o-diphenols to o-quinones [16]. Numerous methods have been used for the immobilization of tyrosinase onto various substrates including carbon paste immobilization, sol–gel immobilization, physical adsorption, Langmuir-Blodgett thin films, electrochemical entrapment of enzyme within conducting polymer or composite matrix etc. [16-18, 19-20].
In this research, biosensor based on screen-printed electrode and tyrosinase has been developed. Iron(II) phthalocyanine has been used as electron mediator. The biosensing properties towards catechin have been studied. Feasibility of the biosensor to detect biologic active compounds from medicinal plant extracts has been evaluated. The catechin content obtained with biosensor has been compared with results obtained by means of Folin-Ciocalteau method.

Materials and Methods

Chemical and solutions

The sources of materials and reagents used were as follows: catechin and iron phthalocyanine from Sigma-Aldrich; tyrosinase (EC 1.14.18.1, from mushroom) was purchased from Sigma. A 46μg·μL^{-1} solution of tyrosinase in buffer phosphate 0.01 M (pH=7) was used for the enzyme immobilization. The buffer was prepared from potassium monobasic and dibasic phosphate salts from Aldrich. The aqueous solutions were prepared using 18 MΩ·cm ultrapure water (Thermo Scientific TKA).

Screen-printed based biosensor

Screen-Printed Electrodes (SPE) were purchased from Dropsens (model DRP-110SWCNT). SPEs constituted by a SWCNTs (single wall carbon nanotubes) working electrode with a surface diameter of 4 mm, carbon as counter electrode and silver/silver chloride electrode as reference one, were used.
The working electrode was modified covering the SPE with a total of 50 μL of FePc solution (10^{-6}M in chloroform) adding 5 μL each step and it was allowed to dry.
The enzyme, tyrosinase (Ty), was immobilized on the above SPE by a casting technique followed by cross-linking. 10μL of 0.01 M phosphate buffer (pH 7.0) containing 46μg·μL^{-1} of enzyme, was added onto SPE surface. After drying, the biosensor was exposed to a 2.5% (v/v) glutaraldehyde solution (in phosphate buffer 0.01M of pH 7) for 20 minutes at room temperature. The enzyme-immobilized electrode was dried at 10ºC and rinsed with phosphate buffer solution thrice to remove any unbound enzyme from the biosensor surface and was further dried at 10ºC and stored at 4ºC.

Apparatus

All electrochemical experiments were preformed using EG&G PARC Model 263 potentiostat/galvanostat interfaced to a PC with Echem software. A three-electrode cell was used. The working electrode, counter electrode and reference electrodes of the screen-printed electrodes were connected directly to the potentiostat via soldered wires. The screen-printed electrodes (SPE) from DropSens (Oviedo, Spain) consisted by a SWCNTs working electrode (4mm diameter), carbon as counter electrode and silver/silver chloride electrode as reference one, were used (see www.dropsens.com). The SPEs were used “as-received” without

activation, chemical or electrochemical cleaning of the working electrode surface before modification.
All the electrochemical experiments were carried out in 0.01 M phosphate buffer solution (PBS) of pH=7 as supporting electrolyte.

Samples
Samples of green tea were obtained from local supermarkets. Amount of 0.1 g samples of green tea were added in 10 mL of hot water (70ºC) in triplicate. For the electrochemical experiments, 50 mL aliquots of these green tea samples were used. For the Folin-Ciocalteau method, the green tea samples were prepared by diluting four times in ultrapure water.

Determination of polyphenols content using Folin–Ciocalteau method
The Folin–Ciocalteau method was performed as described by Singleton and Rossi Jr. [21]. An aliquot of 30 μL of the diluted sample solution was mixed with 150 μL of commercial Folin-Ciocalteau reagent and 450 μL of 20 wt.% sodium carbonate aqueous solution, the final volume was adjust to 3.0 ml with ultrapure water. The color generated was read after about 2 h at room temperature at 735 nm in 1 cm quartz cuvette using a UV-Vis spectrophotometer from Labomed inc. (Model UVD-3000) connected to a PC (software UVWin). A calibration plot of absorbance vs. polyphenol concentration was made using catechin as standard. The polyphenol content in green tea samples was evaluated from the generated absorbance value.

Results and Discussions

The appropriate immobilization of the enzyme in solid substrates is crucial for the development of the biosensors. The structure of the matrix used for Ty immobilization contributes to the maintenance of enzyme functionality. Thus, in first step the viability of the immobilized tyrosinase were carried out by cyclic voltammetry.

Cyclic voltammetry studies
The response towards catechin solution of the biosensor was registered in the range from -0.4 V to +0.7V at a scan rate of 0.1 $V \cdot s^{-1}$ (Figure 1).

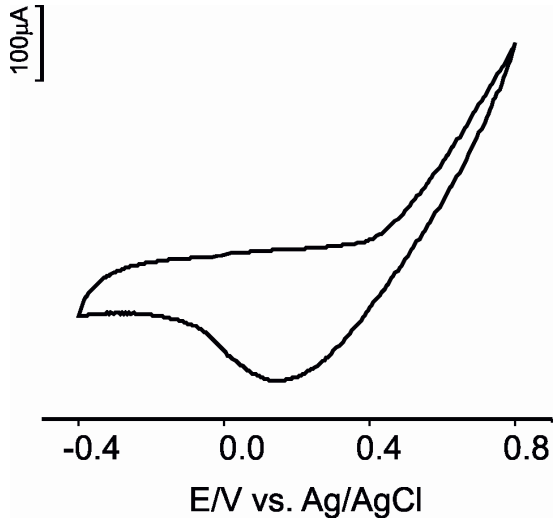

Figure 1: Cyclic voltammogram of biosensor in 10^{-4}M catechin solution (10^{-2}M PBS pH 7.0)

The cyclic voltammogram of biosensor in catechin solution do not show the peaks related with phthalocyanine. As is show in the Figure 1, only the peak corresponding to reduction of the o-quinone enzymatically formed at electrode surface appearing at 0.15V is observed. The presence of reduction peak indicates that the immobilization process retains the biocatalytic activity of tyrosinase immobilized onto solid substrate.

Tyrosinase catalyzes the oxidation of catechin to o-catechin-quinone derivatives, as shown in Eq. 1.

$$\text{Catechin} + \text{Tyrosinase } (O_2) \rightarrow \text{o-Catechin-quinone} + H_2O \quad (1)$$

The generated o-quinones can be reduced electrochemically at low potential without any mediator (Eq. 2).

$$\text{o- Catechin-quinone} + 2H^+ + 2e^- \rightarrow \text{Catechin} \quad (2)$$

Therefore, catechin can be detected by electrochemical reduction of the o-catechin-quinone. If the electron mediator is added in immobilization matrix this have two effects, the lowering of peak potential and increasing of peak current, increasing sensibility of the biosensor. In Figure 2 are presented the functioning scheme of the biosensor and the effect of FePc (electron mediator) in the biosensor response.

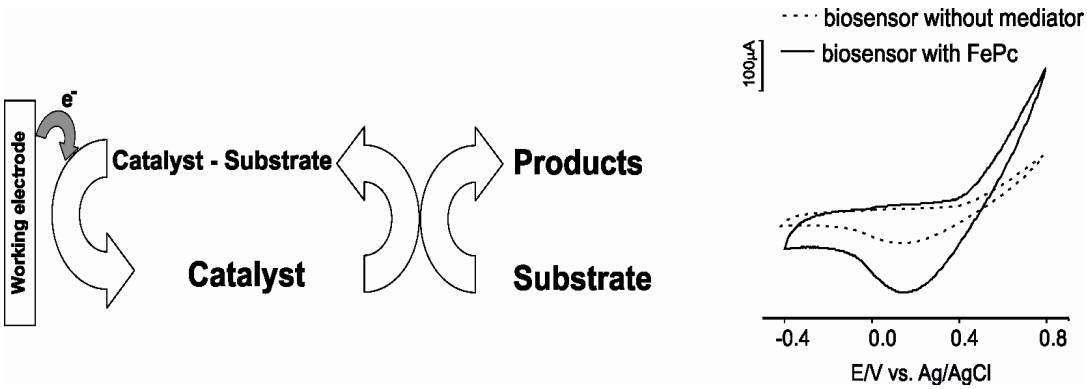

Figure 2. The functioning scheme of the biosensor (left) and the effect of electron mediator in the biosensor response (right)

Optimization of the experimental parameters

The applied potential has an important influence over the biosensor response, because the applied potential contributes to the sensitivity and selectivity of the system [22]. The potential dependence on the biosensor response is shown in Figure 3 using 10^{-4}M catechin in 0.01 M phosphate buffer (pH 7.0).

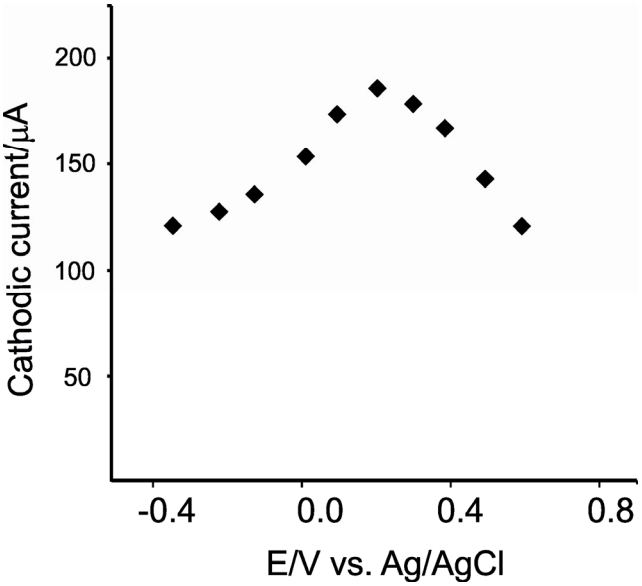

Figure 3: Current - potential dependence in 0.01 M PBS (pH 7.0) and 10^{-4} M catechin under stirring.

The maximum of the signal is obtained at 0.15 V. Therefore, 0.15 V was used as the applied potential. This potential is favorable, since only some chemical compounds expected to be present in samples are reduced at such a low potential [23].
It is well-known that pH is a critical parameter of the enzymatic activity and the stability in aqueous media [24]. The effect of pH in the analysis of catechin with the biosensor is presented in Figure 4.

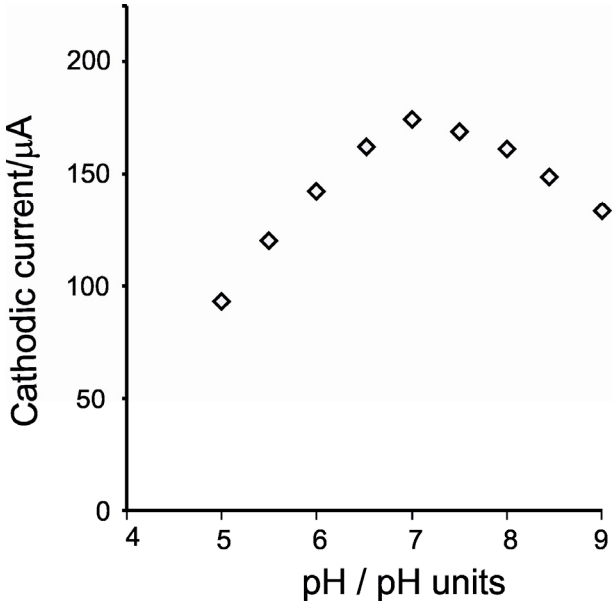

Figure 4: The effect of pH value in biosensor response. Experimental conditions: 0.01 M PBS containing 10^{-4} M catechin. Applied potential: 0.15 V.

The best response was observed at pH 7.0, this value is close to the optimum pH observed for soluble tyrosinase [25]. This behavior shows that the carbonaceous matrix does not affect the optimum pH for the catalytic activity of immobilized tyrosinase, when catechin was used as substrate.

Kinetic of the biosensor

Kinetic studies were performed by registering the cyclic voltammograms of the biosensor at different scan rates, from 0.05 to 0.50 V s^{-1}. The results are presented in Figure 5.

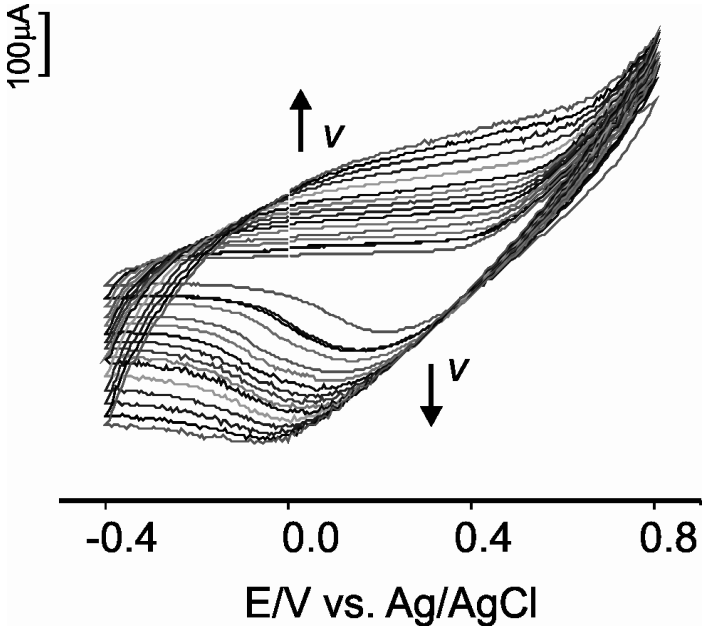

Figure 5: Cyclic voltammograms of biosensor in 10^{-4} M catechin (pH 7.0) registered with different scan rates (0.05–0.50 V s^{-1}).

The peak currents were proportional to scan rates (linear equation of the plot was $y = -24.56x + 155.18$; $R^2 = 0.9967$), pointing to a charge transfer limited process. From the slope of this line and using the Laviron equation (Eq. 3):

$$I_c = n^2F^2\nu A\Gamma/4RT \quad (3)$$

where Γ is the surface coverage (mol cm^{-2}), A is the electrode area (cm^2), ν is the potential scan rate and n, I_c, F, R and T have their usual meanings [26], the total surface coverage could be calculated. The total surface coverage calculated was $8.92 \cdot 10^{-10}$ mol cm^{-2}.

Amperometric response of the biosensor

Figure 6 illustrates a typical amperometric response for the biosensor at 0.15 V after the addition of successive aliquots of catechin to the 0.01 M PBS (pH 7.0) under constant stirring. As observed in the Figure, a well-defined reduction current proportional to the concentration of catechin is observed, which results from the electrochemical reduction of o-catechin-quinone enzymatically formed.

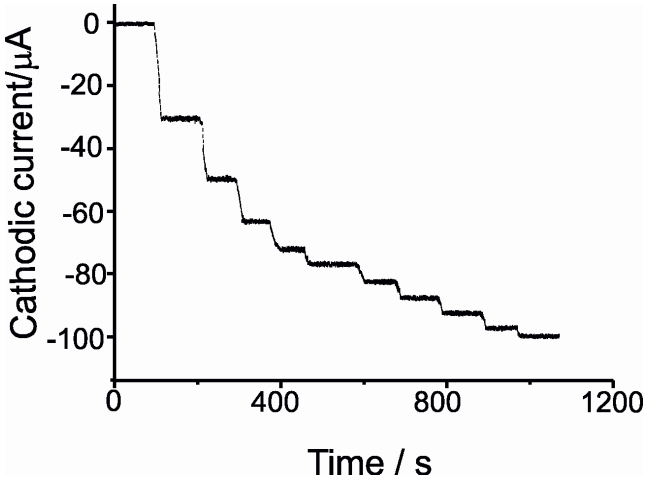

Figure 6: Amperometric response of biosensor to catechin in 0.01 M PBS (pH = 7). Applied potential 0.15 V.

The biosensor achieves 95% of steady-state current in less than 10 s. The response velocity is comparable with than that of 10 s reported in a conducting polymer film [27] and 50 s reported in the pure silica sol–gel matrix [28].

Effect of catechin concentration

Figure 7 shows the relationship between the cathodic current of the biosensor and the catechin concentration in PBS (pH 7.0) at 0.15 V (calibration curve). The response current is linear with catechin concentration in the range from 5 to 250 μM. After that, with supplementary increasing catechin concentration, the cathodic current increases slowly.

The sensitivity of the biosensor is 0.937 μA μM^{-1}. The corresponding detection limits were calculated according to the 3σ/m criterion, where m is the slope of the calibration graph, and σ was estimated as the standard deviation (n = 5) of the voltammetric signals from different solutions of the substrate at the concentration level corresponding to the lowest concentration of the calibration plot. The detection limits calculated were 0.89 μM.

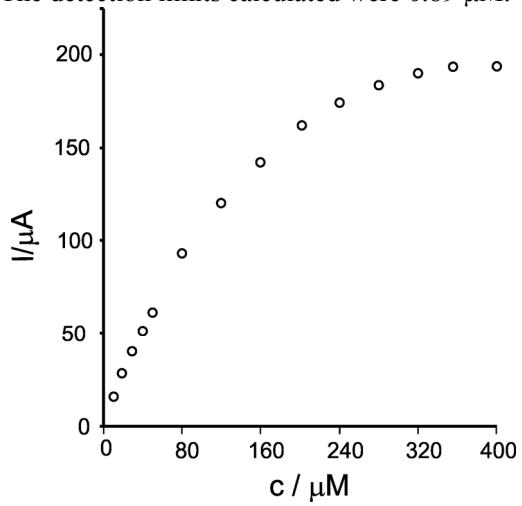

Figure 7: The calibration curve between the cathodic current and the concentration of catechin in PBS (pH 7.0) at 0.15 V.

Application of the biosensor to determination of polyphenols concentration of green tea extracts

Table 1 report the polyphenols content in green tea samples measured with Folin–Ciocalteau spectrophotometric method and proposed biosensor. In the determinations of polyphenols, the catechin was used as standard [29,30]. The analytical performances of two methods are slightly different as demonstrated by the mean values. The values obtained with the biosensor were smaller than that obtained with Folin-Ciocalteau method. These results are consistent with the literature values determined by tyrosinase-based biosensor [29,30]. These values can be explained considering the heterogeneity of natural phenol present in the samples, the majority of them can react with the non-selective phosphotungstate-molybdate reagent of Folin-Ciocalteau [31].

Table 1: Estimated concentration of total polyphenols (as catechin) in green tea samples

	Plant extract		
	Green Tea 1	**Green Tea 2**	**Green Tea 3**
Spectrophotometric method (mg)	98±3.86	51±2.35	19±1.45
Biosensor (mg)	89±2.24	46±2.13	16±1.55

As observed in the Table 1 exists a very good relationship between the results obtains by both methods. Therefore, the biosensor proposed here can be used successfully in real samples analysis.

Conclusions

The proposed tyrosinase biosensor exhibited a good sensitivity and optimal analytical characteristics, which may be attributed to an immobilization procedure. Therefore, the screen-printed SWCNTs modified with FePc, can be utilized as a suitable matrix for the immobilization of tyrosinase. The biosensor is appropriate for the quantitative analysis of polyphenols in natural extracts without pretreatment of the sample in a short analysis time. The experimental results have shown good agreement with the Folin–Ciocalteau method. The possible applications of this biosensor include determination of catechin content in vegetables extracts.

Acknowledgments

This work has benefited from financial support through the 2010 POSDRU/89/1.5/S/52432 project, ORGANIZING THE NATIONAL INTEREST POSTDOCTORAL SCHOOL OF APPLIED BIOTECHNOLOGIES WITH IMPACT ON ROMANIAN BIOECONOMY, project co-financed by the European Social Fund through the Sectoral Operational Programme Human Resources Development 2007-2013.

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