Talanta 83 (2010) 249–255

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

An amperometric biosensor based on peroxidases from *Brassica napus* for the determination of the total polyphenolic content in wine and tea samples

Adrian Marcelo Granero[a], Héctor Fernández[a,*], Elizabeth Agostini[b], María Alicia Zón[a,**]

[a] *Departamento de Química, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Agencia Postal N° 3, 5800-Río Cuarto, Córdoba, Argentina*

[b] *Departamento de Biología Molecular, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Agencia Postal N° 3, 5800-Río Cuarto, Córdoba, Argentina*

ARTICLE INFO

Article history:
Received 11 June 2010
Received in revised form
10 September 2010
Accepted 14 September 2010
Available online 19 September 2010

Keywords:
Amperometric biosensor
Hairy roots
Peroxidase enzymes
Brassica napus
Wines
Teas

ABSTRACT

An amperometric biosensor based on peroxidases from *Brassica napus* hairy roots (PBHR) used to determine the total polyphenolic content in wine and tea samples is proposed by the first time. The method employs carbon paste (CP) electrodes filled up with PBHR, ferrocene (Fc), and multi-walled carbon nanotubes embedded in a mineral oil (MWCNT + MO) at a given composition (PBHR–Fc–MWCNT + MO). The biosensor was covered externally with a dialysis membrane, which was fixed at the electrode body side part with a Teflon laboratory film and an O-ring. Calibration curves obtained from steady-state currents as a function of the concentration of a polyphenolic standard reference compound such as t-resveratrol (t-Res) or caffeic acid (CA) were then used to estimate the total polyphenolic content in real samples. The reproducibility and the repeatability were of 7.0% and 4.1% for t-Res (8.4% and 5.2% for CA), respectively, showing a good biosensor performance. The calibration curves were linear in a concentration range from 0.05 to 52 mg L^{-1} and 0.06 to 69 mg L^{-1} for t-Res and CA, respectively. The lowest polyphenolic compound concentration values measured experimentally for a signal to noise ratio of 3:1 were 0.023 mg L^{-1} and 0.020 mg L^{-1} for t-Res and CA, respectively.

1. Introduction

Polyphenolic compounds are a complex group of substances that have gained enormous attention in the last years, mainly in the analytical chemistry field, because they have important health properties [1–3] and antioxidant activity [4,5].

Polyphenolic compounds are in skin grapes, mainly in seeds and epidermal cells. The amount and quality of polyphenolic compounds in grapes depend on the variety of wine grape, the weather, the soil, and the farming practices [6]. The main polyphenolic compounds in wines with antioxidant activity are derivative from phenolic and cinnamic acids, tirosyne stilbenes, flavonoids and procianidins [4]. The total concentration of phenolic compounds in red wines varies from 1800 to 4060 mg L^{-1} (expressed as mg L^{-1} of galic acid, with an average value of 2570 mg L^{-1}). A lesser concentration of polyphenolic compounds is found in white wines, varying between 160 and 330 mg L^{-1}, with an average value of 240 mg L^{-1} [7].

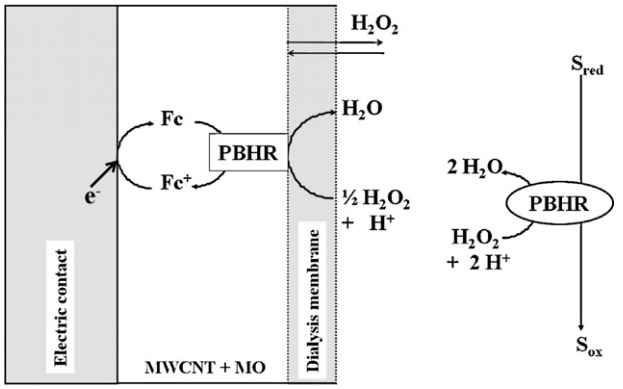
Tea represents a major source of antioxidants since it is the most widely consumed beverage worldwide. It has beneficial health effects, given its anti-carcinogenic, anti-teratogenic and antimicrobial properties [8–13]. Different tea varieties and extracts usually present in food have been reported to prevent pathogenic agents, such as *Escherichia coli*, *Salmonella* sp. and *Staphylococcus aureus* [13–16].

At present, several methods are available for the analysis of polyphenolic compounds. Most of the methods are based on separation techniques such as chromatography or capillary electrophoresis with various detection systems [17–20] as well as techniques that do not involve separation steps, i.e., vanillin–HCl, n-butanol–HCl and the Folin–Ciocalteu colorimetric method [21]. The Folin–Ciocalteu method, based on the reaction of phenolic compounds with a colorimetric reagent, allows the determination of phenolic and/or polyphenolic compounds in the visible region of the electromagnetic spectrum [22]. However, this spectrophotometric approach yields an overestimation of total polyphenolic content (TPC) [4].

Biosensors have been proposed as an efficient analytical tool for the determination of polyphenolic compounds, exhibiting advantages such as the minimal preparation of the sample, selectivity, sensitivity, reproducibility, rapid time of response and simple use for continuous on-site analysis [23,5,24–29].

* Corresponding author. .
** Corresponding author. Tel.: +54 358 4676233; fax: +54 358 4676233.
E-mail addresses: agranero@exa.unrc.edu.ar (A.M. Granero), hfernandez@exa.unrc.edu.ar (H. Fernández), eagostini@exa.unrc.edu.ar (E. Agostini), azon@exa.unrc.edu.ar (M.A. Zón).

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.09.016



Scheme 1. Two-stage reaction scheme for detecting phenolic compounds using carbon paste electrodes filled up with peroxidases obtained from *Brassica napus* hairy roots (PBHR), ferrocene (Fc) and, multi-walled carbon nanotubes embedded in a mineral oil (MWCNT + MO). S_{red} and S_{ox} are the reduced and oxidized forms of the phenolic compound, respectively.

In this article, we propose an amperometric biosensor based on peroxidases obtained from *Brassica napus* hairy roots (PBHR) to determine the TPC in wine and tea samples. The method employs carbon paste (CP) electrodes filled up with PBHR, ferrocene (Fc), and multi-walled carbon nanotubes embedded with a mineral oil (MWCNT + MO) at a given composition (PBHR–Fc–MWCNT + MO). The biosensor was covered externally with a dialysis membrane (Scheme 1), which was fixed at the electrode body side part with a Teflon laboratory film and an O-ring. It is well known that phenolic and/or polyphenolic compounds can work as electron-donors for peroxidases in the catalytic reduction of H_2O_2 [30,31]. This approach allows detecting the decrease in H_2O_2 concentration in a solution after the oxidation of phenolic and/or polyphenolic compounds produced by the PBHR in the presence of H_2O_2, given that PBHR acts in cascade in the solution and the electrode surface. The separation of the electrode surface from the solution by a semipermeable membrane allows minimizing the electrical noises as well as the fouling of the electrode surface. The latter effect can be produced by the polymerization of oxidized phenolic compounds, which can interfere with electrochemical measurements.

2. Materials and methods

2.1. Reagents and materials

B. napus hairy roots, obtained "in vitro" in our laboratory according to a procedure previously described by Agostini et al. [32], were used as the enzyme source.

Hydrogen peroxide (30%, v/v), pH 7.00 phosphate buffer solutions (PBS), sodium acetate, acetic acid, NaCl, HCl, NaOH and Na_2CO_3 were Merck p.a.; trans-resveratrol (t-Res), caffeic acid (CA), ferrocene (Fc), o-dianisidine, sodium hydrogen sulfite, glucose and ascorbic acid 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 λ_{max} = 240 nm (ε = 43.6 mol^{-1} L cm^{-1}) [33]. Stock solutions of t-Res and CA were prepared in H_2O and kept at a temperature of 4 °C.

MWCNT + MO was obtained by mixing different amounts of MWCNT (Sigma, outer diameter 30–50 nm, inner diameter 5–15 nm, length = 0.5–200 μm) with MO (Sigma) in order to optimize the final best composition. 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 body side part with a Teflon laboratory film and an O-ring.

2.2. Total extraction and purification of enzymes and determination of the peroxidase activity

Hairy roots were homogenized in a mortar with 10 mmol L^{-1} pH 4.00 sodium acetate/acetic acid buffer, containing 1 mol L^{-1} NaCl (1 g fresh roots weight per 3 mL of buffer) at 4 °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 peroxidases and determine total peroxidase activity.

The purification of peroxidases was performed by molecular exclusion chromatography on Sephacryl S-200-HR (SIGMA) columns. Samples of 2.5 mL of TPE, previously dialyzed, were loaded on a Sephacryl S-200-HR column (2 cm × 32 cm) equilibrated with a pH 7.00 phosphate buffer 20 mmol L^{-1}. The column was washed with 100 mL from this buffer at a flow rate of 1 mL min^{-1} controlled with a peristaltic pump (LKB 2232 Microperpex). Fractions of 5 mL were collected and monitored to determine their peroxidase activity using a qualitative method proposed by Forchetti and Tigier [34]. Fractions containing most peroxidases were mixed and lyophilized before they were used. These enzymes presented a peroxidase activity of 280 IU per solid mg and they were labeled as PBHR.

The total peroxidase activity was determined with o-dianisidine as substrate [35] and expressed in international unit (IU), defined as the amount of enzyme forming 1 mmol of product in 1 min under the experimental conditions employed.

2.3. Real samples

The TPC was estimated in different wine and tea samples. We used four red and two white wines, two green, one red and two black teas. They were purchased from a local supermarket and produced in Argentina.

Wine samples did not require any pre-treatment. The tea samples were treated following this procedure: 2 g of tea were put in 200 mL of H_2O and the mixture was boiled for 4 min and then filtered [36].

The TPC estimation in wine and tea samples was carried out by interpolation of the corresponding amperometric signals with the corresponding calibration curves constructed with t-Res and CA stock solutions.

For comparison purposes, samples of wine and tea were also analyzed with the espectrophotometric method using the Folin–Ciocalteu reagent [37]. Standard solutions were prepared by adding 4.5 mL of 2% (w/v) Na_2CO_3 aqueous solution and the corresponding amount of wine or tea. Then, 230 μL of the Folin–Ciocalteu reagent (1:1 v/v in methanol) were added after 2 min and leveled in a 5 mL volumetric flask. After being allowed to react for 30 min, protected from light, the absorbances of the standards (t-Res and CA) were measured at λ = 750 nm [22]. The TPC in samples was expressed as mg of t-Res or CA per liter of the sample.

2.4. Instruments and experimental measurements

Cyclic voltammograms and amperometric measurements were performed with an epsilon (BAS) potentiostat controlled by electrochemical analysis software. Electrochemical measurements were carried out in a 2 mL Pyrex cell. The working electrode was a CP disk of 1.6 mm diameter obtained from Bioanalytical System (BAS), Inc. The counter electrode was a platinum foil of large area (~2 cm^2). An aqueous SCE was used as the reference electrode. Aliquots of 5 and 10 μL were added to the electrochemical cell for the determination of TPC in red wines and white wines and tea samples, respectively. Amperometric measurements were performed at a potential of −0.050 V *vs* SCE in solutions stirred at 1600 rpm. This operational applied potential was previously optimized by Granero et al. [38].