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First report on electrocatalytic oxidation of oxytetracycline by horse radish peroxidase: Application in developing a biosensor to oxytetracycline determination

Javad Ghodsi, Amir Abbas Rafati*, Yalda Shoja

Department of Physical Chemistry, Faculty of Chemistry, Bu-Ali Sina University, P.O. Box 65174, Hamedan, Iran

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ABSTRACT

This work describes the development of an easy to fabricate biosensor for determination of oxytetracycline (OTC) in basis of electrocatalytically oxidation of OTC by horse radish peroxidase (HRP) in the presence of H_2O_2. This work is first report on determination of OTC by enzymatic modified electrode. Developed electrode was fabricated by modifying of CPE with HRP and MWCNTs. This electrode provides sensitive and selective determination for OTC. Detection limit, linear range and sensitivity for OTC determination were completely satisfactory and were about 35 nM, 15 μM–1.5 mM and 18.74 μA/mM, respectively. Applicability of developed electrode was verified with successfully determination of OTC in pharmaceutical and biologic samples. Also developed electrode was showed the selective determination of OTC in presence of uric and folic acid.

1. Introduction

Antibiotics are a special group of pharmaceuticals used to control infection diseases in human and veterinary medicine [1]. Tetracyclines (TCs) are widely used antibiotics to prevent bacterial infections in livestock and increase their growth rate [2]. They are broad-spectrum antibiotics that prevent activity of both Gram-positive and Gram-negative bacteria. They are produced during fermentation process by *Streptomyces rimosus*. They are extensively used for the curing of infectious diseases in veterinary and human medicine, but also as additives in animal feeds to promote growth [3]. Oxytetracycline (OTC), a member of the tetracycline family of antibiotics, is a common antibiotic with a broad range of activity and a low cost. It widely used in swine and animal farming. Its low absorption on oral administration causes the contamination of swine manure by unmetabolized material [4,5] and makes it as most frequently detected tetracyclines in water bodies and sediments around the world [6]. It is verified the residue of OTC in the environment is potentially harmful. It is reported that OTC can prevent the bioactivity of various microorganisms [5,7]. OTC abuse can seriously affect human health. Abuse of TCs in animals farming results in accumulation of antibiotics in food products, including milk, meat, and chicken eggs [2]. Ultimately, this accumulation can seriously affect the human health. Antibiotic residues in milk can trigger the emergence of antibiotic-resistant bacteria [8]. Antibiotics residual in environment is so important that several countries have set a maximum residue limits (MRLs) for many food products and extensive efforts have been made to develop a sensing system for the enhanced detection of antibiotics in contaminated food products [2]. According to these reasons, the developing of accurate and reliable method to detect the OTC in environments has particular importance. Several methods including fluorometery [9,10], capillary zone electrophoresis [11], potentiometry [12], high performance liquid chromatography [13–16], near infrared spectroscopy [17], UV–vis spectrophotometric [18] and reversed phase liquid chromatography [3] have been reported for determination of OTC in different environments.

However, there are only a few reports on the electrochemical measurement of OTC [19,20], and there is no reported work about enzymatic electrochemical sensor for determination of OTC. As far as we know, rare paper reports the direct determination of OTC based on rapid, selective and sensitive electrochemical method [21].

This work provides a selective, sensitive and repeatable method to determine the OTC with proper applicability in real samples. This electrochemical method is based on electrocatalytically oxidation of phenolic functional group of OTC by HRP in presence of H_2O_2. Several works have been reported on phenolic compounds

* Corresponding author.
E-mail addresses: aa.rafati@basu.ac.ir, rafati.aa@yahoo.com (A.A. Rafati).

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determination by using of peroxidase activity of the HRP [22–25] but so far no report on measurements of OTC by any enzyme modified sensor has been reported. This biosensor was easily fabricated by modification of CPE with HRP and MWCNTs. HRP modified sensors for phenolics have been attracted many attention because of their high sensitivity and selectivity [26–30] and MWCNTs, other electrode modifier in this work, was applied in many electrochemical sensors for their interesting electrical, mechanical and catalytical properties [31–33]. Developed biosensor requires little time to fabricate and its analytical characteristics in OTC determination are very satisfactory in compared to other previously reported methods.

2. Experimental

2.1. Materials

Peroxidase from horseradish (EC.1.11.1.7, 250 U mg^{-1}) was purchased from Sigma–Aldrich and was used as received. MWCNTs with the average diameter of 20–60 nm were purchased from Neutrino Co. (Iran). Oxytetracycline raw material was from Darou Pakhsh Co. (Iran) as gift and OTC tablet 100 mg was purchased from Damloran Razak Pharma Co. (Iran).

All other reagents were of analytical grade. Phosphate buffer solutions (PB) (50 mM) were prepared from H_3PO_4, NaH_2PO_4 and Na_2HPO_4 and pH values were adjusted with HCl and NaOH solutions. All phosphate buffer used in experiments was in pH 7. The solutions were prepared with deionized water and deoxygenated by bubbling high purity (99.99%) nitrogen gas through them for 15 min prior to the experiments. All experiments were carried out at room temperature.

2.2. Apparatus

Voltammetric measurements were performed with a three-electrode system, including the HRP/MWCNTs/CPE as working electrode, an Ag/AgCl (3.0 M KCl) as reference electrode, and a platinum foil as counter electrode. Voltammetric measurements were done using a computer-controlled μ-Autolab modular electrochemical system (PGSTAT101, The Netherlands), driven with NOVA Software (upgrade 1.10). Potential scan rate for all voltammetric measurements was 50 mV/s.

2.3. Electrode fabrication

MWCNTs/HRP modified carbon paste electrode was initially prepared by homogenizing of 20 mg of graphite powder, 20 μl of mineral oil, 5 mg of MWCNTs and 50 μl of HRP/PB solution (10 mg/ml) in a small glass oven and allowed to evaporate the HRP/PB solvent then it completely mixed for several minutes to produce the final paste. The obtained paste was packed into a tip of 1 ml insulin plastic syringe and a cooper wire was inserted to obtain the external electric contact. Finally electrode surface must be polished with piece of soft paper to next electrochemical experiments.

2.4. Methods and procedures

Firstly, for finding optimized amount for H_2O_2 added to analyte, PB solutions containing 1 mM of OTC and different amounts of H_2O_2 (50–1100 μM) were prepared and investigated by DPV voltammograms with scanning potential from 0.4 to 0.9 V and potential scan rate of 100 mV/s (this potential range and scan rate were applied for all DPV tests in this work). H_2O_2 concentration corresponding to maximum anodic current was regarded as optimized amount for H_2O_2.

Afterward, for optimizing of HRP amount applied in electrode modification, eight CPE were modified with same amounts of graphite powder (20 mg), mineral oil (20 μl), MWCNTs (5 mg) and different volumes of HRP solution (10 mg/ml) from 5 to 150 μl. These modified electrodes applied as work electrodes in DPV measurements of 1 mM of OTC and 300 μM of H_2O_2 in PB.

For comparison of electrode response between current modified electrode and some other incomplete modified electrodes, four electrodes were fabricated. These electrodes include (1) CPE (20 mg of graphite powder + 20 μl of mineral oil), (2) MWCNTs/CPE (20 mg of graphite powder + 20 μl of mineral oil + 5 mg of MWCNTs) and (3) HRP/CPE (20 mg of graphite powder + 20 μl of mineral oil + 50 ml of HRP 10 mg/ml). All three mentioned electrodes with main electrode (20 mg of graphite powder + 20 μl of mineral oil + 5 mg of MWCNTs + 50 ml of HRP 10 mg/ml) applied as work electrode in DPV and CV measurements of 300 μM of H_2O_2 and OTC in PB.

For investigating of pH effect on developed electrode response, eight PB containing 300 μM of H_2O_2 and OTC with pHs of 3–8 were prepared (pH adjusted by addition of H_3PO_4 0.1 M and NaOH 0.1 to PB) and then these prepared PBs was investigated by DPV and then anodic peak current of OTC was plotted vs. pH.

Another experiment was performed for evaluating of storage time. A freshly fabricated modified electrode was regarded and applied in DPV measurements of a fixed PB containing 300 μM of H_2O_2 and OTC. DPV measurement was repeated every day for 3 weeks and electrode in this period was kept in 4 °C.

Applicability of modified electrode was investigated with OTC measurements in serum and tablet samples. For preparation of tablet samples, several OTC tablet 100 mg were completely milled and dissolved in PB to reach concentration of 0.1 M and then DPV measurements were carried out and OTC concentration was calculated by standard addition method. Recovery was calculated by following formula:

$$100 + \left(\frac{\text{expected value} - \text{The mean of three measured values}}{\text{expected value}} \times 100\right)$$

For serum sample preparation, bovine serum was diluted forty times in PB and then stock solution of OTC was added to serum solution and DPV measurements were performed. OTC concentration was calculated by standard addition with applying above-mentioned formula.

3. Results and discussion

In many works about phenolic compounds determination with peroxidase enzymes modified electrodes, firstly activated hem of enzyme (with present H_2O_2) oxidizes the phenolic compound to its related quinine (oxidized form of phenolic). Then, quinine can be reduced to initial phenolic form at electrode surface and created reduction current is directly proportional to phenolic concentration. But in this work, applied HRP acts as electrocatalyst for oxidation of OTC in electrode surface that results an oxidative peak current that linearly related to OTC amount in vast concentration range. As showed in Scheme 1a, OTC molecule has a phenolic hydroxyl in its chemical structure that can be oxidized according to electrochemical reaction showed in Scheme 2b. Proposed mechanism for electrocatalytic oxidation of OTC at CPE surface was showed in Scheme 2. As can be seen in Scheme 2, HRP was initially activated (the hem of enzyme was oxidized) by H_2O_2 in analyte solution then activated HRP can easily oxidized OTC and reduced enzyme was oxidized back again at electrode surface which caused the oxidation current. Indeed HRP acts as electrocatalyst for OTC oxidation at electrode surface. All electrochemical experiments in this work are based on oxidation peak current due to electrocatalytically oxidation of OTC at CNT/HRP modified CPE surface. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were