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Tyrosinase/laccase bienzyme biosensor for amperometric determination of phenolic compounds

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ABSTRACT

Tyrosinase/laccase bienzyme biosensor for amperometric determination of phenolic compounds was constructed. Enzymes were immobilized in titania gel matrix. The obtained biosensor was successfully used for determination of 2,6-dimethoxyphenol, 4-tertbutylcatechol, 4-methylcatechol, 3-chlorophenol and catechol. The highest sensitivity and the widest linear range were noticed for catechol, 234 mA L mol^{-1} and 2.0×10^{-7}–3.2×10^{-5} mol/L, respectively. Detection limit for catechol, at signal-to-noise ratio of 3 was 1.3×10^{-7} mol/L.

1. Introduction

Phenolic compounds, widely occurring in the environment, belong to organic pollutants which are highly toxic, carcinogenic and allergenic. Many efforts have been made to develop the simple and effective procedures of the determination of phenols.

A number of methods have been reported to analyze the content of phenols. Chromatographic methods and spectrophotometry have been employed for that purpose [1]. Those methods are expensive, time-consuming, sometimes require preconcentration or extraction steps, and are inadequate for in situ monitoring. Most often electrochemical methods have been used for determination of phenolic compounds. Particularly amperometric biosensors proved to be suitable for phenols determination because of good selectivity, low cost, potential for miniaturization and automation. The biosensors most sensitive to phenols are those based on tyrosinase, copper containing polyphenol oxidize [[2] and references therein]. Tyrosinase catalyses the oxidation of monophenols by molecular oxygen to form *o*-biphenols, which are subsequently oxidized to *o*-quinones [3–8]. Research on biosensors has also been carried out with laccase [2,10–13], cuproprotein belonging to blue polyphenol oxidizes [14]. Laccase catalyses the oxidation of *ortho*- and *para*- diphenols, aminophenols, polyphenols and polyamines [15]. In a typical laccase reaction a phenolic substrate is subjected to one-electron oxidation, giving rise to an aryloxyradical [16]. This active species can be converted to a quinone in the second stage of oxidation. Quinones can be electrochemically reduced to allow convenient low potential detection of the phenolic compounds [4]. The enzymatic electrode has an advantage over direct electrochemical oxidation of phenol because of the low potential of detection (0.0 to 0.2 V versus Ag/AgCl in the former case as compared to +0.8 to +0.95 V versus Ag/AgCl in the later case), since at higher potentials the enzymatically produced quinones polymerize which is responsible for the fouling of electrode [[4] and references therein]. A comparison of analytical characteristics towards catechol for some tyrosinase and laccase biosensors described in the literature is shown in Table 1. Only few papers focused on simultaneous application of two enzymes: tyrosinase and laccase for the determination of phenols were published [17–20]. Freire et al. reported the application of a dual amperometric enzyme-based biosensor device (two separate enzyme-based electrodes, with tyrosinase and laccase) for the resolution of binary mixtures of phenolic compounds with the use of multivariate calibration [17]. A screen-printed four-electrode sensor based on immobilization of laccase, peroxidase, tyrosinase in the same array was developed for monitoring of phenols [18]. Marko–Varga and coworkers proposed, among others, the enzyme-based amperometric sensor with co-immobilized tyrosinase and laccase [19]. Its application in flow injection and column liquid chromatographic systems allowed the detection of phenolic compounds on sub-μg/L levels. Flow injection analysis of phenols at mM levels was performed based on a graphite electrode modified with covalently co-immobilized tyrosinase and laccase [20].

In the present paper a bienzyme biosensor based on tyrosinase and laccase co-immobilized in titania gel matrix was proposed. Gels prepared by the sol–gel method are attractive matrixes for the immobilization of biomolecules, e.g. enzymes, because they can be

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Table 1
A comparison of analytical characteristics towards catechol for tyrosinase and laccase biosensors reported in the literature

Electrode	Sensitivity	Linear range [mol/L]	LOD [mol/L]	Stability	Ref.
Tyrosinase immobilized in Silicate/Nafion composite film	200 mA L mol^{-1}	1×10^{-6}–1×10^{-4}	3.5×10^{-7}	Retained 74% of initial activity after 14 days of storage	[3]
Tyrosinase covalent immobilized onto copolymer poly(*N*-3-aminopropyl pyrrole-co-pyrrole) film	3.46 mA L mol^{-1}	1.6×10^{-6}–1.2×10^{-4}	1.2×10^{-6}	Retained 80% of the enzyme activity for 4 months of storage	[4]
Tyrosinase layer-by-layer immobilized on latex particles	150 mA L $mol^{-1}cm^{-2}$	2×10^{-6}–2.0×10^{-5}	n.r.	Not reported	[5]
Tyrosinase entrapped in polyacrylamide microgels	469.3 mA L $mol^{-1}cm^{-2}$	5.0×10^{-7}–2.4×10^{-5}	3.0×10^{-7}	Not reported	[6]
Tyrosinase immobilized within Os-complex functionalized electrodeposition polymer	6.10 mA L mol^{-1}	Not reported	1×10^{-8}	Not reported	[7]
Tyrosinase immobilized onto graphite-Teflon composite electrode modified with gold nanoparticles	746 mA L mol^{-1}	1.0×10^{-8}–8.0×10^{-6}	3×10^{-9}	39 days without apparently loss of enzyme activity	[8]
Tyrosinase immobilized on a Sonogel–Carbon matrix	82.5 mA L mol^{-1}	Not reported	6.4×10^{-8}	Not reported	[9]
Recombinant laccases immobilized using bovine serum and glutaraldehyd onto graphite electrode (GE)	3.8 mA L mol^{-1}	1×10^{-6}–1.3×10^{-5}	n.r.	Not reported	[2]
Cross-linked enzyme crystals of laccase embedded in polyvinylpropylidone gel	Not reported	5×10^{-5}–1.0×10^{-3}	n.r.	40% of initial activity was lost after 30 cycles of measurements	[10]
Laccase immobilized in the matrix of carbon nano-tubes-chitosan composite	Not reported	1.2×10^{-6}–3.0×10^{-5}	6.6×10^{-7}	1% decrease of current response after 15 days of storage	[11]
Laccase covalently immobilized on a hydrophilic matrix (flow system)	91 mA L mol^{-1}	0–5×10^{-4}	2×10^{-8}–3×10^{-8} (for various phenols)	Sensitivity of the biosensor was constant for more than 100 working days	[12]

prepared under ambient conditions and can retain the catalytic activities of enzymes [21–23]. Titania gel is a solution of choice in the case of the construction of a biosensor because titania is known to be hydrophilic and biocompatible, and the titania gels may be easily prepared by the sol–gel procedure. At the same time the simultaneous application of two enzymes may extend the substrate spectrum of a biosensor. The functioning of proposed biosensor was checked to be useful for the detection of following phenolic compounds: 2,6-dimethoxyphenol, 4-tertbutylcatechol, 4-methylcatechol, 3-chlorophenol and catechol. The storage stability and operational activity of the sensor were evaluated.

To our best knowledge there are no reports on determination of phenolic compounds using bienzyme biosensors with tyrosinase and laccase immobilized in titania sol–gel matrix.

2. Experimental

2.1. Apparatus

Cyclic voltammetry was applied to establish the working potential range of the enzymatic electrode. Voltammetric measurements were performed using potentiostat Electrochemical Interface 1286, Schlumberger-Solartron (UK). Amperometric experiments were carried out using the potentiostat EP-21 Elpan (Poland). A conventional three-electrode system was employed with the enzyme electrode as a working electrode, a platinum wire as the counter electrode, and the saturated calomel electrode (SCE) as a reference electrode. All experiments were performed at the temperature of 25 °C. For voltammetric experiments the solution (phosphate buffer) was purged from oxygen by bubbling with laboratory grade argon. Amperometric experiments were carried out in electrochemical cell containing phosphate buffer at pH=6, under constant stirring with magnetic bar and under free access of air. The enzyme electrode worked at a potential 0.0 V vs. SCE. Signals were recorded after 3 min in buffer and after 5 min in phenolic solutions. Exemplary chronoamperometric response of bienzyme electrode in phosphate buffer solution at pH=6 and in 1.5×10^{-5} mol/L catechol are presented in Fig. 1.

2.2. Reagents

Tyrosinase from mushroom was purchased from Sigma. Laccase, *Cerrena Unicolor*, was obtained in the Biochemistry Department, Maria Curie Skłodowska University, Lublin, Poland. Acetone and 2-propanol were purchased from POCh (Poland); nitric acid, hydrochloric acid and ammonia were from Chempur (Poland); $N_2HPO_4{\cdot}H_2O$ and KH_2PO_4 were from Merck. Precursor, titanium isopropoxide, was bought from Fluka Chemie GmbH (Switzerland). Phenolic compounds were purchased as follows: catechol from BDH Chemical (UK), 2,6-dimetoxyphenol and 4-methylcatechol were from Sigma-Aldrich (Germany), 4-tertbutylcatechol and 3-chlorophenol from Fluka Chemie GmbH (Switzerland). All chemicals were analytical grade and were used as received. Solutions were prepared using double-distilled water. Paraffin wax used for impregnation of carbon electrodes was produced by BDH Chemical. Carbon rods of the diameter of 0.6 cm, used in spectrographic analysis, and produced by Electrokarbon Topolcany (Slovak Republic) were used for the construction of the electrodes.

2.3. Construction of the carbon electrodes

Carbon electrodes were impregnated in paraffin wax for 15 min at 80 °C, and placed in teflon holder with stainless steel wire as a current lead fastened to the end of the electrode. The working surface of electrode was polished first with emery papers (from Struers, Germany), starting with the 180 and ending with the 2000 grades, followed by Al_2O_3, finally rinsed with distilled water, and sonificated for 5 min in the following media: distilled water, nitric acid (1:1), ammonia water, saturated solution of ascorbic acid and acetone. Electrode was rinsed with distilled water after each sonification and finally dried at room temperature.

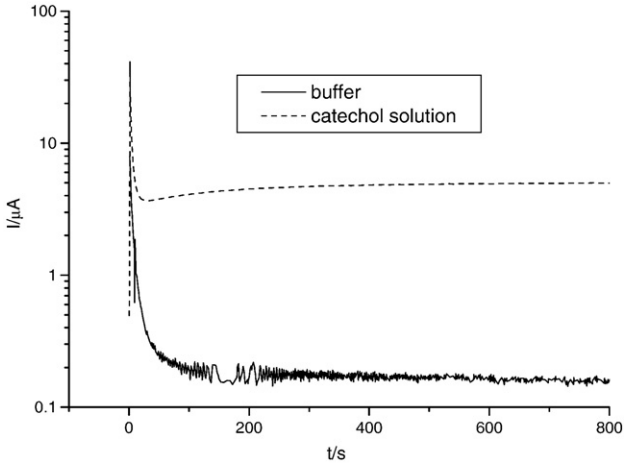

Fig. 1. Exemplary chronoamperometric response of bienzyme electrode in phosphate buffer solution at pH=6 and 1.5×10^{-5} mol/L catechol. Potential 0.0 mV vs. SCE.