Biosensing L-DOPA with laccase-based enzyme electrodes: a comparative study

M. E. Shukri[1], N. Dimcheva[1,2]*

*[1]Laboratory on Biosensors, Centre of Technologies, University of Plovdiv "Paisii Hilendarski", 21 „Kostaki Peev"
Str., Plovdiv, Bulgaria*
[2]Faculty of Chemistry, Department of. Physical Chemistry, 24, "Tsar Assen" Str., Plovdiv 4000, Bulgaria

The focus of the present work is to develop and optimize an electrochemical laccase-based biosensor for the determination of 3,4-dihydroxyphenyl-alanine, also known as L-DOPA. The biosensor was assembled on a conventional glassy carbon electrode, the surface of which was covered with laccase enzyme retained under a thin Nafion™ membrane. The enzymes used for this purpose were isolated and purified from the white-rot basidiomycetes (*Trametes sp.*) *Trametes pubescens* and *Trametes versicolor*. Although biochemically similar, the two enzymes demonstrated some differences in their affinity not only using 3,4-dihydroxyphenyl-L-alanine as enzyme substrate, but also when catecholamines such as dopamine and L-epinephrine were used. A range of electrochemical techniques were used for the study, such as cyclic voltammetry, chronoamperometry, and electrochemical impedance spectroscopy. Experiments were performed using buffers with different pH and applying various substrate concentrations. Activity and sensitivity of the two alternative laccase – based biosensors were compared by means of chronoamperometry. The biosensor produced on the basis of *Trametes pubescens* laccase, operating in citrate buffer with pH 4 proved to be more suitable than the one based on laccase purified from *Trametes versicolor* for biosensing L-DOPA.

Keywords: Laccase from basidiomycetes, electrochemical biosensor, L-DOPA.

INTRODUCTION

Due to its ability to prevent Parkinson's disease which is characterized by lack of dopamine in nerve cells, L-DOPA has gathered considerable attention. L-DOPA can be oxidized in cells by the enzyme tyrosinase, which reaction is responsible for the formation of the skin pigment melanin [1]. Reactive oxygen species (ROS) include hydroxyl radical (HO•), superoxide anion (O_2•-), and hydrogen peroxide (H_2O_2) can be produced during these reactions. L-DOPA is also a dopamine precursor, which may undergo enzyme-catalysed decarboxylation to form the neurotransmitter [2].

Laccases (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductases) are multi-copper glycoproteins that are widely found in nature [3]. Through a reaction mechanism catalyzed by radicals, they oxidize a variety of aromatic and non-aromatic compounds *via* molecular oxygen [4]. Laccases show catalytic activity toward ortho- and para-diphenol groups, including mono-, di-, and poly-phenols, aminophenols, methoxyphenols, aromatic amines and ascorbate [5]. This class of multicopper oxidases can oxidize a variety of substrates by using oxygen as an electron acceptor. Neither reactive oxygen species (ROS), which are dangerous, nor partially reduced O_2 species are released by multicopper oxidases (MCOs), which catalyse enzymatic conversion of O_2 directly to H_2O *via* a 4-electron mechanism [6].

Laccases belong to a rare class of metalloproteins that are capable of exchanging electrons with the underlying electrode surface and thus to reduce electrocatalytically oxygen to water [7, 8]. These enzymes have been detected in a variety of organisms, such as bacteria, fungi, plants, and insects. Most common laccase producers are the wood rotting basidiomycetes, e.g. *Trametes versicolor, Trametes pubescens, Trametes hirsute, Trametes ochracea, Trametes villosa, Trametes gallica, Cerena maxima, Coriolposis polyzona, Lentinus tigrinus, Pleurotus eryngii*, etc. [9, 10].

A number of analytical methods have been used for the determination of L-DOPA, such as high-performance liquid chromatography [11], electrochemiluminescence [12], spectrophotometry [13], flow injection analysis, capillary zone electrophoresis [14], and fluorescence methods [15]. Despite the high sensitivity offered by these methods, a number of drawbacks have been noted, including complexity of the equipment, need for highly-trained personnel, lengthy testing times, and high costs of analysis. In contrast to these approaches, electrochemical techniques have

* To whom all correspondence should be sent:
E-mail: *ninadd@uni-plovdiv.bg*

attracted increasing interest because of their ease of use, affordability, high sensitivity, and quick response time [16].

Biosensing L-DOPA is usually implemented by means of tyrosinase-based enzyme electrodes [17], operating within a large potential window, where undesired electrochemical transformations may take place as well. On the other side, there is only a limited number of reports on biosensing L-DOPA by means of a laccase-based enzyme electrode.

Thus, the purpose of the present work is to compare the catalytic performance of two laccase enzymes, purified from Basidiomycetes of *Trametes sp.* during the catalytic oxidation of L-DOPA, assayed electrochemically with the aim to select the best performing enzyme to be used in the construction of a biosensing system capable of discriminating between catecholamines and L-DOPA.

EXPERIMENTAL

Laccase (E.C. 1.10.3.2) enzymes from *Trametes versicolor* (Fluka) and *Trametes pubescens* (a generous gift from Prof. Dr. Roland Ludwig, Department of Food Science and Technology, BOKU–University of Natural Resources and Life Science) were with homogeneous specific activity of 23 U mg^{-1} and 46 U mg^{-1}, respectively. One unit of enzyme is defined as the oxidation of 1.0 μmol of ABTS per min at pH 4 and 30 °C. Laccases were used as solutions in 0.05 M sodium-citrate buffer, pH = 4, with concentrations of 50 mg/mL and 20 mg/mL, respectively.

The enzyme electrodes were prepared on the basis of commercial glassy carbon electrodes (2 mm diameter, Metrohm, Utrecht, The Netherlands). Prior to modification, the electrodes were cleaned by ultrasonication in ultrapure water for two minutes, after which they were polished with 0.05 μm alumina slurry on a polishing cloth (Kulzer, Hanau, Germany), water-rinsed and cleaned by ultrasonication in ultrapure water again.

Enzyme immobilization was implemented as follows: 2 μl of enzyme solution (20 mg/ml *Trametes pubescens* laccase or 50 mg/ml *Trametes versicolor* laccase) were drop-cast on the electrode surface. Then, a 4 μl drop of 0.2% Nafion 117 was applied. The surface was allowed to dry at room temperature.

After measurements, the enzyme electrodes were rinsed with bidistilled water and refrigerated at 4°C when not in use. The regeneration of the working enzyme electrodes can be done after the mechanical removal of the enzyme-polymer layer *via* polishing procedure and following the above steps.

All electrochemical experiments were performed in a conventional three-electrode cell with working volume of 10 mL, connected to a computer-controlled electrochemical workstation, Autolab PGSTAT 302 N (Metrohm-Autolab, Utrecht, The Netherlands) equipped with NOVA 2.1.6 software. Unmodified or modified glassy carbon electrode was used as working, Ag|AgCl, sat. KCl (Metrohm, Utrecht, The Netherlands)—as a reference—and a platinum foil as an auxiliary electrode. If not otherwise specified, all reported potentials were referred to Ag|AgCl, sat. KCl electrode.

Cyclic voltammetry was run at a scan rate of 5 mV.s^{-1}. Chronoamperometric detection was tested at three different potentials (-0.1 V, -0.2 V and -0.3 V). The chronoamperometric detection was carried out with a rotating disc electrode at a rotation speed of 500 rpm .

Volt-ampere curves were obtained by adding aliquots of L-DOPA with a concentration of 10 mM to 10 ml of citrate buffer (pH 4 and pH 4.5) in the following order - 3×20 μl, 3×50 μl, 3×100 μl, 3×200 μl, 3×500 μl, 3×1000 μl.

Electrochemical impedance spectroscopy (EIS) was performed in a conventional 3-electrode setup, in 0.1M KCl containing 5 mM of K$_4$[Fe(CN)$_6$]/K$_3$[Fe(CN)$_6$] as redox probe over the range of frequencies from 100 kHz to 1 Hz.

RESULTS AND DISCUSSION

Enzyme immobilization on the electrode surface is of key importance when aiming at a long-lasting stability of the resulting bioelectrode. For this purpose, laccase was retained on the electrode surface under a semi-permeable membrane of ionomer Nafion, possessing negatively-charged groups. To examine the specific properties of the electrode-electrolyte interface of the studied system, electrochemical impedance spectroscopy (EIS) – a non-destructive alternating current technique, was applied.

Complex plane plots of the impedance for unmodified glassy carbon electrode and for the same electrode covered with polymer layer entrapping either of the two laccases are depicted in Fig. 1. Nyquist plot for the bare glassy carbon electrode consists of a semi-circle region characteristic for the charge-transfer resistance over the high frequencies range, followed by a very short linear region tilted at *ca*. 45° typical for Warburg impedance (indicative for diffusion control over the redox process) arising over the low-frequencies range. The EIS-spectra of glassy carbon electrodes bearing immobilized laccases are extremely similar to the one of the bare glassy carbon. The only difference is that the radii of

the semi-circle regions of the two enzyme-bearing electrodes are larger than the one observed in the EIS spectrum of bare glassy carbon electrode. This finding is associated with an increased charge transfer resistance, indicative for partly hampered penetration of the redox probe that is most probably a result from the increased thickness of the enzyme-confining polymer layer.
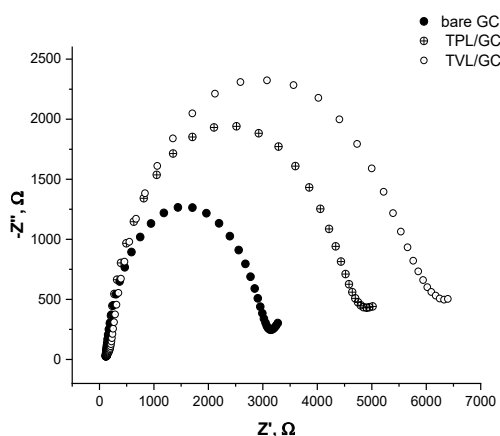

Fig. 1. Impedance spectra of: (•) bare glassy carbon electrode (GCE); (⊕) *Trametes pubescens* laccase immobilized on GCE; (o) *Trametes versicolor* laccase immobilized on GCE.

Further, the electrochemical activity of the two types of laccase-based enzyme electrodes was studied by cyclic voltammetry (CV). Comparison of the CVs of the enzyme electrode recorded in the absence and the presence of oxygen (Fig. 2) reveals laccase-catalyzed electrochemical reduction of dissolved oxygen. On the CVs recorded with a low scan rate in deaerated buffer (Fig. 2, black line) no peaks are detectable on both forward and reverse scans. On the other hand, the CV recorded in aerated buffer solution (Fig. 2, red line), shows a clearly expressed reductive wave starting below 0.1 V, which is due to the electrochemical reduction of dissolved oxygen catalyzed by the immobilized enzyme. As it was underlined in the Introduction, laccase is one of the few oxidoreductases capable of exchanging electrons with underlying electrode surfaces without the need for additional electron shuttles (mediators), with the efficiency of the electrical communication controlled by both the enzyme orientation and the distance between its active site and the electrode surface. It is hypothesized that the negatively charged Nafion membrane formed during the immobilization process electrostatically repulses the negatively charged laccase active site, this way orienting the enzyme in a conformation favorable for electron exchange with the underlying electrode surface,

which becomes obvious in the presence of molecular oxygen. Therefore, the voltammetric studies revealed its ability for performing bio-electrocatalytic oxygen reduction to water thus confirming that the chosen way of enzyme immobilization yields an enzyme electrode with electrocatalytically active enzyme.

The preliminary experiments have shown that in the presence of substituted phenols, the current of the oxygen reduction increases proportionally to the concentration of the added substance, which phenomenon is also known as mediated electron transfer (MET). In this respect, the electrode response of both types of laccase-based biosensors was studied at constant potentials over the range from -0.1 to -0.3 V *vs.* Ag|AgCl, sat. KCl upon addition of aliquots of 3,4-dihydroxy phenylalanine stock solutions to the working medium. The resulting dependencies of the electrode response on substrate concentration were found to follow Michaelis type kinetics, with the slopes of the linear parts of the plots being dependent on both the potential at which the bioelectrode was poised, and pH of the operating medium (Fig. 3). For both laccase-based electrodes, higher electrode sensitivities were detected at pH = 4.0, and therefore all further studies were performed at this pH.
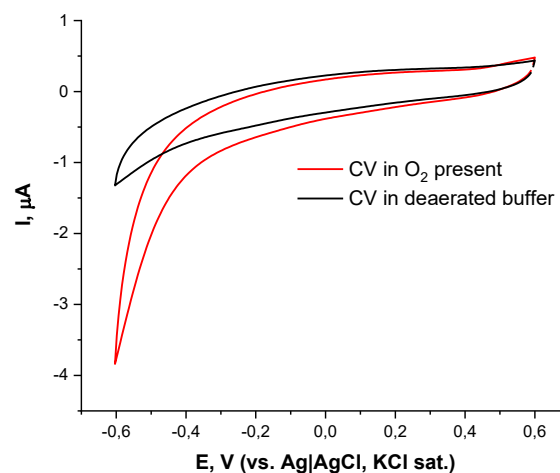

Fig. 2. Cyclic voltammograms of laccase electrode recorded in the presence (red) and in the absence (black) of dissolved O_2, electrolyte – citrate buffer, pH = 4.

Differences were noted in the variation of the electrode sensitivity with changing the polarization potential. For the electrode with immobilized laccase from *Trametes versicolor*, electrode sensitivity remains practically constant at applied potentials of -0.1 and -0.2 V, then slightly decreases at -0.3 V (Fig. 3A, black line), whilst for the other type of laccase-based electrode (*Trametes pubescens*) there is a visible sensitivity increase at an applied potential

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of -0.2 V (Fig. 3B, black line) as compared to -0.1 V and -0.3 V. It is worth mentioning that there is a big difference in the sensitivity values for the two types of enzyme electrodes – the one for *Trametes versicolor* laccase–based electrode is almost twice as low as the sensitivity of the other type of enzyme electrode.
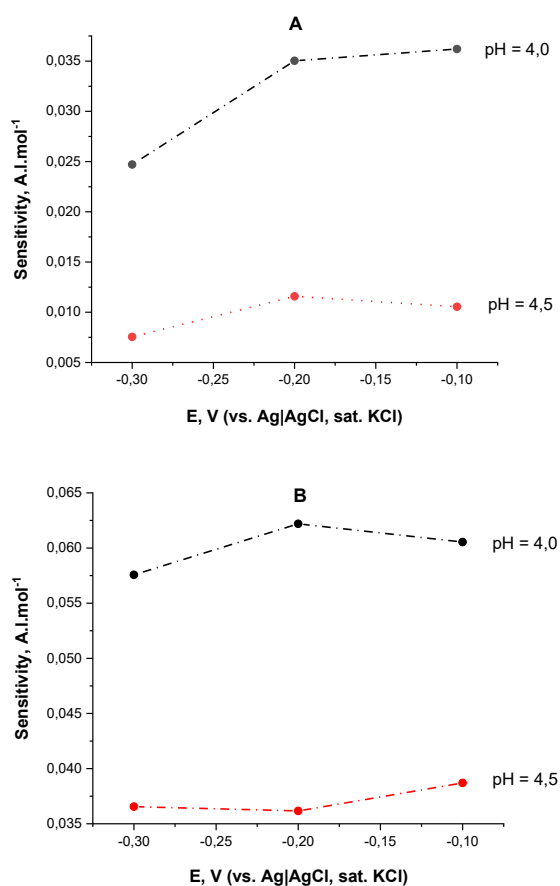

Fig. 3. Electrode sensitivities (slopes of the calibration graphs) as a function of electrode polarization potential at two different pHs of the medium for the enzyme electrode with immobilized laccase from: A) *Trametes versicolor*; B) *Trametes pubescens*.

In Fig. 4 are depicted the dependencies of the current density on the concentration of L-DOPA determined with the two types of laccase-based electrodes under the working conditions selected as optimal: working potential of -0.2 V and pH = 4.0. The similarity in the shape of the curves is obvious – both electrode types manifest hyperbolic trends of the dependencies of the electrode response on the substrate concentration. However, the apparent kinetic constants for the two immobilized laccases, determined from non-linear regression of the experimental data, show noticeable differences. The apparent Michaelis constant for immobilized laccase (*Trametes versicolor*, Fig. 4B) was found to be: $K_M^{app} = 0.89 \pm 0.012$ mM and $V_{max}^{app} = 329.8 \pm 32.8$

μA cm⁻², whilst for the immobilized laccase from *Trametes pubescens* (Fig. 4A), the apparent kinetic constants were calculated as $K_M^{app} = 1.77 \pm 0.76$ mM and $V_{max}^{app} = 273.3 \pm 7.0$ μA cm⁻². These differences in the apparent kinetic constants of the two identically immobilized enzymes suggest that despite biochemical similarity of the two laccases – the one isolated from *Trametes versicolor*, and the one purified from *Trametes pubescens*, there are noticeable differences in enzyme affinity towards L-DOPA, with *Trametes versicolor* laccase possessing greater enzyme affinity towards this particular substrate thus shortening the linear dynamic range within which the substrate can be determined.
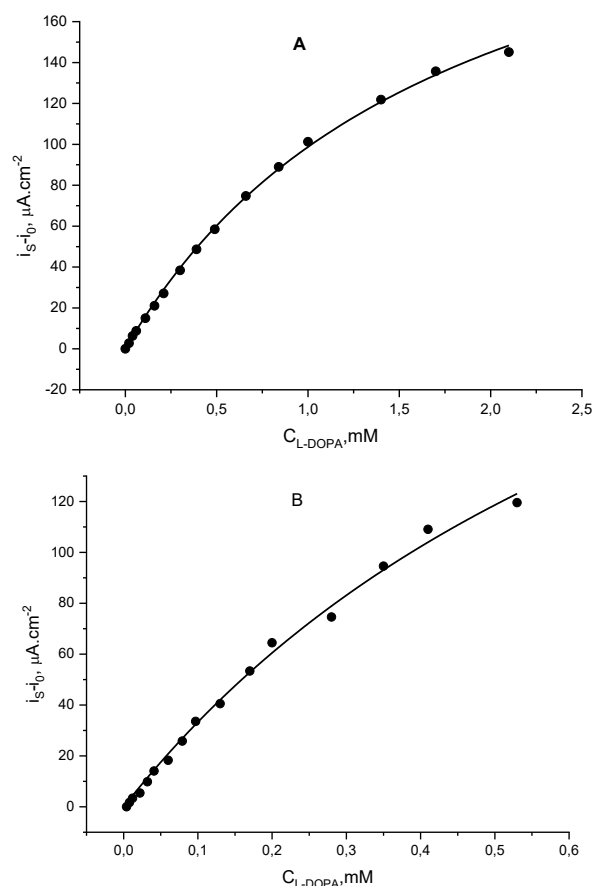

Fig. 4. Dependence of the current density of the biosensor on the L-DOPA concentration for: A) *Trametes pubescens*; B) *Trametes versicolor*; working potential E = -0.2 V.

Operational stability of the produced laccase electrode was tested over a period of 5 days and the resulting remaining activity as a function of number of measurements performed with the same electrode is depicted in Fig. 5. It could be seen that over the first four measurements the electrode gradually loses ca 7% of its activity. It retains about 30% of its initial activity after 10 measurements (on the day 2 of preparation), that can be explained with loss of catalytic activity of the enzyme due to both enzyme

leakage in the maintenance buffer and enzyme inactivation due to continuous exposure at room temperature.

The two types of laccase electrodes were tested for interference from L-ascorbic acid, dopamine, and L-epinephrine, since L-ascorbate is a usual attendant of real samples where L-DOPA is to be assayed, and the two catecholamines (dopamine and L-epinephrine) can be eventually present in pharmaceuticals containing the analyte under study.
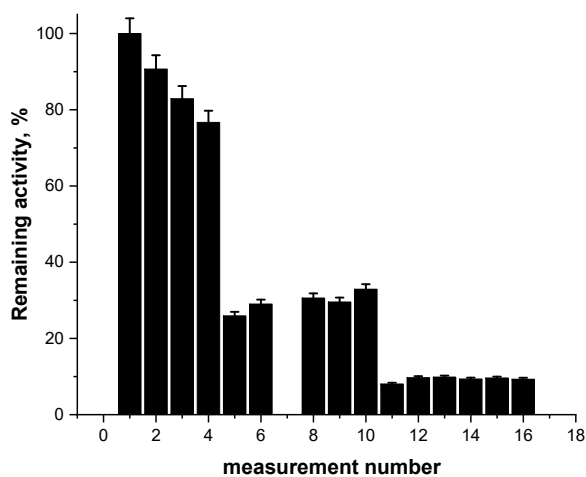

Fig. 5. Remaining electrode activity as a function of measurement number.

The results indicate that under the working potential, the interference effect of L-ascorbate on the assay of L-DOPA would not be substantial, however the presence of catecholamines may largely compromise the analysis of 3,4-dihydroxy phenylalanine.

Based on the above considerations, it can be concluded that laccase from *Trametes pubescens* is more suitable for the development of an enzyme electrode for the determination of 3,4- dihydroxy phenylalanine because of: i) wider linear dynamic range; ii) facilitated substrate penetration through the enzyme layer due to lower enzyme load in the electrode-covering membrane, and iii) greater electrode sensitivity.

REFERENCES

1. M. Dai, T. Huang, L. Chao, Y. Tan, C. Chen, W. Meng, Q. Xie, *RSC Advances*, **6**, 17016 (2016).
2. J. A. Best, H. F. Nijhout, M. C. Reed, *Theoretical Biology and Medical Modelling*, **6**, 21 (2009).
3. L. Arregui, M. Ayala, X. Gómez-Gil, G. Gutiérrez-Soto, C. E. Hernández-Luna, M. Herrera de los Santos, L. Levin, A. Rojo-Domínguez, D. Romero-Martínez, M. C. N. Saparrat, M. A. Trujillo-Roldán, N. A. Valdez-Cruz, *Microbial Cell Factories*, **18**, 200 (2019).
4. M. Tišma, P. Žnidaršič-Plazl, I. Plazl, B. Zelić, D. Vasić-Račkic, *Chemical and Biochemical Engineering Quarterly*, **22**, 307 (2008).
5. M. M. Rodríguez-Delgado, G. S. Alemán-Nava, J. M. Rodríguez-Delgado, G. Dieck-Assad, S. O. Martínez-Chapa, D. Barceló, R. Parra, *TrAC Trends in Analytical Chemistry*, **74**, 21 (2015).
6. G. Janusz, A. Czuryło, M. Frąc, B. Rola, J. Sulej, A. Pawlik, M. Siwulski, J. Rogalski, *World Journal of Microbiology and Biotechnology*, **31**, 121 (2015).
7. D. M. Ivnitski, C. Khripin, H. R. Luckarift, G. R. Johnson, P. Atanassov, *Electrochim. Acta*, **55**, 7385 (2010).
8. M. Pimpilova, K. Kamarska, N. Dimcheva, *Biosensors*, **12**, 719 (2022).
9. V. Madhavi, S. S. Lele, *BioResources*, **4**, 1694 (2009).
10. Q. Wang, L. Ding, C. Zhu, *Biotechnology & Biotechnological Equipment*, **32**, 1477 (2018).
11. T. Dhanani, R. Singh, S. Shah, P. Kumari, S. Kumar, *Green Chemistry Letters and Reviews*, **8**, 43 (2015).
12. N. Mirzanasiri, M. Hosseini, B. Larijani, H. Rashedi, *Analytical and Bioanalytical Electrochemistry*, **10**, 147 (2018).
13. M. F. Abdel-Ghany, L. A. Hussein, M. F. Ayad, M. M. Youssef, *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, **171**, 236 (2017).
14. L. Zhang, G. Chen, Q. Hu, Y. Fang, *Analytica Chimica Acta*, 431, 287 (2001).
15. A. Moslehipour, A. Bigdeli, F. Ghasemi, M. R. Hormozi-Nezhad, *Microchemical Journal*, **148**, 591 (2019).
16. R. G. State, J.K.O.O.S.F. van Staden, *Electrochemical Science Advances*, **2,** e2100040 (2022).
17. F. Mollamohammadi, H. Faridnouri, E. N. Zare, *Biosensors*, **13**, 562 (2023).