Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1806-2563
ISSN on-line: 1807-8664
Doi: 10.4025/actascitechnol.v37i2.19660

Effect of mediator added to modified paste carbon electrodes with immobilized laccase from *Aspergillus oryzae*

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ABSTRACT. Carbon paste electrodes based on the immobilization of laccase from *Aspergillus oryzae* were developed and voltammetric measurements were performed to evaluate the amperometric response. The 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) functions as substrate and mediator for the laccase enzyme. Electrodes were modified in two different conditions: without mediator (EPC/laccase) and with mediator (EPC/laccase/ABTS). The addition of ABTS as a mediator increased eight-fold the amperometric response. The electrode was sensitive to pH variation with best response at pH 4.0. Studies on different concentrations of laccase and ABTS at different pH rates revealed that the composition 187 U mL^{-1} in laccase and 200 μL of ABTS obtained the highest amperometric response. The carbon paste electrode modified with ABTS proved to be a good base for the immobilization of the laccase enzyme. Moreover, it is easy to manufacture and inexpensive to produce a modified electrode with potential application in biosensors.

Keywords: cyclic voltammetry, immobilization, ABTS, biosensors.

Efeito da adição de mediador em eletrodos de pasta de carbono contendo lacase de *Aspergillus oryzae*

RESUMO. Foram desenvolvidos eletrodos de pasta de carbono baseados na imobilização de lacase de *Aspergillus oryzae* e foram realizadas medidas voltamétricas para verificar a resposta amperométrica. O sal de 2,2′- azino-bis 3-etilbenzotiazolina-6-sulfonato (ABTS) funciona como mediador e substrato para a enzima lacase. Foram modificados eletrodos em duas condições especificas: sem o mediador (EPC/lacase) e com o mediador (EPC/lacase/ABTS). A adição do ABTS como mediador aumentou a resposta amperométrica em oito vezes. Este eletrodo se mostrou sensível à variação de pH obtendo melhor resposta em pH 4,0. Estudos que envolvem diferentes concentrações de lacase e ABTS, em diferentes valores de pH revelaram que a composição 187 U mL^{-1} de lacase e 200 μL de ABTS obteve a maior resposta amperométrica. A pasta de carbono se mostrou um bom suporte para imobilização da enzima lacase, além de ser de fácil fabricação e de baixo custo para possível aplicação como biossensor.

Palavras-chave: voltametria cíclica, imobilização, ABTS, bissensores.

Introduction

Biosensors may be defined as modified sensors with biological material attached to the surface of the electrode. They are promising tools for detecting compounds since they complement existing analytic methods, such as chromatography and spectroscopy, by making them faster, more versatile, sensitive and selective (CLARK JR.; LYONS, 1962; JAROSZ-WILKOLAZKA et al., 2004; CHAWLA et al., 2012). It is expected that the establishment of these devices could be analytically employed in medicine, agriculture, food safety, environmental monitoring and industries. However, high costs and technological barriers make the transfer of technology to these sectors slow and limited when compared to the number of publications and patents on the technology developed (LUONG et al., 2008).

Among the materials which may be used in the construction of biosensors, carbon-based materials, such as carbon paste, are widely used. These materials are very inert and have a wide-ranging potential in aqueous solution, the crystalline structure that enables residual low current and high signal-to-noise, with low costs. (AHUJA et al., 2007). Carbon paste consists of a mixture of graphite powder and a binder, such as Nujol, Uvasol, paraffin, Bromonaphthalene which must be electroinactive, chemically inert, water-immiscible,

with low volatility and low amounts of impurities. (AHAMMAD et al., 2009; ZIMA et al., 2009).

Laccase (EC1.10.3.2), an oxidase that contains copper in its structure and widely distributed in fungi, plants and some bacteria, is a potential enzyme for biosensor development for phenols and polyphenols. It is able to oxidize polyphenols, anilines, and benzothiazoles with a concomitant reduction of oxygen in water (JAROSZ-WILKOLAZKA et al., 2004; RICCI; PALLESCHI, 2005; FERNANDES et al., 2008). Laccase has been widely used in biosensors to determine phenolic compounds (FREIRE et al., 2001; KULYS; VIDZIUNAITE, 2003; DI FUSCO et al., 2010; RAWAL et al., 2012).

In amperometric biosensors using mediators, the sensitivity of these devices may be increased by assisting the transfer of electrons between the enzyme and the electrode surface (MARQUES; YAMANAKA, 2008). Substrates with reversible electrochemical behavior and chemical stability are called mediators. The 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) functions as a substrate and as a mediator for laccase enzymes (LIU et al., 2006). The incorporation of these compounds to the electrode has been made by adsorption, occlusion or addition in polymer films on carbon paste (MARQUES; YAMANAKA, 2008).

Current assay investigates the immobilization of laccase from *Aspergillus oryzae* on carbon paste electrodes. The electrode is also modified with ABTS as a direct mediator in the carbon paste. The response of the electrode is examined in terms of electrolyte pH, enzyme activity and ABTS concentration in the paste.

Material and methods

Reagents and solutions

All reagents used in this work were of analytical grade (PA) and used without prior purification. All solutions were prepared with deionized and ultra-purified water. KCl (0.3 mol L^{-1}) and ABTS (20 mmol L^{-1}) solutions were prepared for the study of electrochemical electrodes. Graphite powder (Fluka) and commercial mineral oil (Nujol Union Chemical) were used in the preparation of carbon paste, whilst 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) was purchase from Sigma-Aldrich. The *Aspergillus oryzae* fungus laccase was obtained from a sample of the product NOVOZYM 51003®, kindly donated by the Novozymes Company.

Equipment and electrodes

Cyclic voltammetry measurements were obtained with a MQPG-01 potentiostat. An electrode Ag/AgCl was used as reference and a platinum coil as an auxiliary electrode. The carbon paste electrode, modified with immobilized laccase, was the working electrode. All voltammetric measurements were made in a 25 mL-glass electrochemical cell under the following conditions: scan rate equals to 20 m V s^{-1} and potential varying between 0 and +1.0 V.

Determination of enzyme activity of laccase

The activity of laccase from *Aspergillus oryzae* was determined by oxidation of ABTS as substrate contained in the solution at a concentration of 50 mmol L^{-1}, and pH 3.00 (Mac'Ilvaine, 120 mmol L^{-1} buffer) at 50°C, as described by Barbosa et al. (1996). The reaction time was 5 min. and the activity was determined by measuring the absorption at 420 nm. The unit of laccase activity was defined as the number of ABTS micromols oxidized per minute per ml of the enzyme solution. Laccase activity in the enzyme solution received from the manufacturer was equal to 937 U mL^{-1}, which is consistent with that provided by the supplier (1000 U mL^{-1}).

Preparation of carbon paste electrode

The carbon paste electrode (EPC/laccase) was prepared by macerating 150 mg of graphite powder, 50 μL of mineral oil and laccase from *Aspergillus oryzae*, during 40 min. After the preparation of the paste, it was placed at the tip of 1mL-plastic syringes, modified with a copper wire, coupled to the piston connected to the external electrical contacting a section of the graphite. The contact between the copper wire and the section of graphite was improved by a conductive paint (Condumax plus). Two working electrodes were prepared, or rather, with the enzyme (EPC/laccase) and without the enzyme (EPC). The two electrodes were stored at 4ºC. In the case of electrodes with mediator ABTS, different volumes of ABTS 20 mmol L^{-1} solutions were added to the paste carbon and macerated for 30 min.

Electrolyte

The ABTS mediator was added to 0.3 mol L^{-1} KCl solution until a concentration of 20 mmol L^{-1} was obtained. Different pH rates (2.0-7.0) for the electrolyte (20 mmol $^{o}L^{-1}$ ABTS + 0.3 mol L^{-1} KCl) were studied. pH was attained by adding HCl to the solution.

Effect of laccase concentration

The effect of laccase (*Aspergillus oryzae,* Novozym® 51003) concentration was studied using different volumes 50, 100, 150 and 200 µL of laccase solution in carbon paste, whose enzymatic activity respectively corresponded to 47, 94, 141 and 187 U mL^{-1}.

Results and discussion

Laccase is a very interesting enzyme since it belongs to the group of redox enzymes that show direct heterogeneous electron transfer at electrodes. The enzyme catalyzes the oxidation of the organic substrates, mostly phenolic compounds, by molecular oxygen in homogeneous solutions. (YAROPOLOV et al., 1994) The substrate functions as soluble electron donor, since it is reduced in a mediated electron transfer process. In such a mechanism the substrate penetrates the active site of the enzyme. The substrate is oxidized in a single electron oxidation step (YAROPOLOV et al., 1994). However, the product of this reaction could be electrochemically active and could be re-reduced at electrode surface. Substrates that present reversible electrochemical behavior and chemical stability may be referred to as mediators. The recycling process of the mediator causes an increase in the sensitivity of detection if compared with direct electrochemical oxidation. Normally, this process is used for the development of amperometric biosensors (FREIRE et al., 2001). The affinity of laccase enzyme and ABTS substrate/mediator is well known. The ABTS presents an electrochemical reversibility while it has been shown that ABTS oxidation provides a pair of redox peaks of ABTS (LIU et al., 2006).

Effect of laccase (*Aspergillus oryzae*; NOVOZYM 51003®) concentration and pH on the response of carbon paste electrode

The effect of laccase concentration added to a mixture of carbon paste has been studied since the rate of a reaction catalyzed by the enzyme depends directly on its concentration. Further, pH has a great influence on the enzymatic reactions. Enzymes have optimal pH when the activity and speed of reactions are maximal. Therefore, current analysis studied the effect of pH between 3.0 and 7.0 on the response of the carbon paste electrode modified with laccase.

Figure 1 compares current responses measured in µA when the potential was set at 0.5 V as a function of pH for different electrodes. Amount in volume and, consequently, in activity varied.

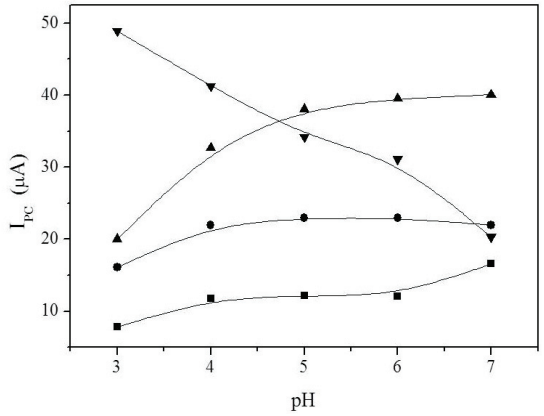

Figure 1. Dependence of amperometric response (I_{pc}) at + 0.5 V for a carbon paste modified electrode with different concentrations of laccase (-■- 47; -♦- 94; -▲- 141; and - ▼- 187 U mL^{-1}) at different pH rates. Supporting electrolyte: ABTS 20 m mol L^{-1} and KCl 0.3 $molL^{-1}$.

The effect of different amounts of immobilized laccase showed a higher sensitivity when 187 U mL^{-1} of enzyme were added to the mixture of carbon paste at pH 3.0. It has been observed that, in the case of a large amount of enzyme in carbon paste, current decreased with increasing pH rates of the enzyme concentration. A similar behavior was reported by Liu et al. (2006) for the immobilized laccase in the composite carbon nanotube/chitosan. This behavior is also similar to the free enzyme in the solution. However, when the amount of enzyme decreases in carbon paste, pH increase raises peak current rates. In such conditions the enzyme may have probably found a micro-environmental where it could behave differently.

As expected, the results for electrodes prepared with enzymes with activity 47 and 94 U mL^{-1} had the lowest response of the electrode. Considering the electrode with 47 U mL^{-1}, the response dependence with pH had a slight augmentation when pH varied from 4.0 to 7.0. However, in the case of electrodes containing 94 U mL^{-1} enzyme, the current rates remained in a constant effect between pH 4.0 and pH 7.0. With regard to 141 U mL^{-1}, an increase in the electrode response has been reported with increasing pH of the electrolyte solution.

Optimal pH for laccase activity depends on the type of substrate (DESAI; NITYANAND, 2011). In the case of phenols, fungal laccase has generally optimal activity in the pH range between 3.0 and 7.0 (BOLLAG; LEONOWICZ, 1984). An optimal pH response for biosensors using immobilized laccase in other systems has been reported between 4.0 for laccase immobilized in chitosan microspheres (FERNANDES et al., 2008) and 6.0 for the immobilized laccase in a complex

copper/chitosan/carbon nanotubes/polyaniline (CHAWLA et al., 2011).

Effect of mediator on electrodes constructed with laccase (*Aspergillus Oryzae*; Novozym 51003®)

Several recent studies on enzyme immobilization have focused on different materials and methods for attaching the biologic agent to an insoluble support, basically as biosensors (ZHANG et al., 2000; FERNÁNDEZ-FERNÁNDEZ et al., 2012). Carbon-based materials have been successfully used in the construction of these devices (JAROSZ-WILKOLAZKA et al., 2004; LIU et al., 2006; SANTHIAGO; VIEIRA, 2007; FERNANDES et al., 2008; OLIVEIRA et al., 2013). In current study, the carbon paste modified with laccase from the fungus *Aspergillus oryzae* was tested as a modified electrode and a mediator was added to the medium. Although the substrate ABTS is used to determine the activity of laccase, in current assay it was added to the mixture of carbon paste modified by the enzyme to be analyzed as a mediator and its effect on the voltammetric response of the electrode evaluated. Figure 2 shows the voltammogram of the carbon paste electrode with (EPC/Laccase/ABTS) and without (EPC/Laccase) mediator ABTS. Results show that no evidence of oxidation peaks or reduction is extant without the intermediary. In other words, the carbon paste does not interfere with the response of electrodes constructed. However, with the addition of ABTS (50 mmol L^{-1}) as a mediator, the amperometric response obtained had an 8-fold increase. Considered as the first mediator referred to laccase, ABTS salt has been applied together with this enzyme in a mixture of carbon paste, a procedure that has resulted in better stability and sensitivity for biosensors (JOHANNES; MAJCHERCZYK, 2000). The oxidation of ABTS by laccase occurs in two steps, which correspond to two oxidation peaks obtained in the cyclic voltammogram of the EPC/laccase/ABTS electrode. Initially, the laccase triggers ABTS oxidation that occurs with the formation of radical cation $ABTS^{+\bullet}$ with a correspondence with first peak oxidation E_{pcI}, in cyclic voltammetry profile, in approximately + 0.17 V; the second oxidation peak, E_{pcII}, represents the oxidation of the radical cation forming the di-cation $ABTS^{2+}$ in approximately + 0.73 V (Figure 2).

Results show a good response to the laccase-mediator system applied to the modified electrode. They reveal that, by cyclic voltammetry, the role of the mediator ABTS, acting in oxidation reactions, improves the mechanism of electron transfer in surface electrode (FABRINI et al., 2002; LIU et al., 2006; ODACI et al., 2006; LIU; DONG, 2008).

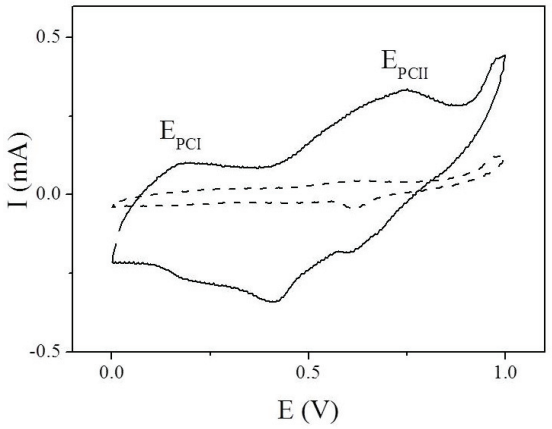

Figure 2. Cyclic voltammograms obtained for carbon paste electrodes modified by electrode with laccase: (—) with mediator (EPC/LAC/ABTS) and (- - -) without mediator (EPC/LAC). Supporting electrolyte: ABTS 20 mmol L^{-1} and KCl 0.3 mol L^{-1} at pH 2.0. s = 20 mV s^{-1}.

Effect of the ratio between the quantities of ABTS/laccase added to carbon paste

The performance of modified carbon paste electrodes depends on the concentration of laccase used for electrode preparation, mediator amount and pH of the supporting electrolyte solution. When preparing graphite electrodes modified with *Aspergillus oryzae* laccase, concentrations of the enzyme solution were pipetted and mixed with carbon paste. The amount of enzyme was converted into activity and selected as 94, 147 and 188 U. In a second step, ABTS was added to the paste and 100, 150 and 200 µL were chosen. The electrodes were tested in solutions containing ABTS 20 mmol L^{-1} and KCl 0.3 mol L^{-1}, at different pH rates: 2.0, 3.0 and 4.0.

These experiments demonstrated how the electrode behaved when the carbon paste had several compositions (which indicated a standard) and the electrolyte had its pH adjusted to different values.

Three factor arrangements engendered 27 different combinations (Figure 3). It may be observed that the higher amperometric response occurred when the highest amount of mediator and the biggest enzyme activity were added to the carbon paste mixture, in other words, 200 µL ABTS and 188 U mL^{-1} laccase.

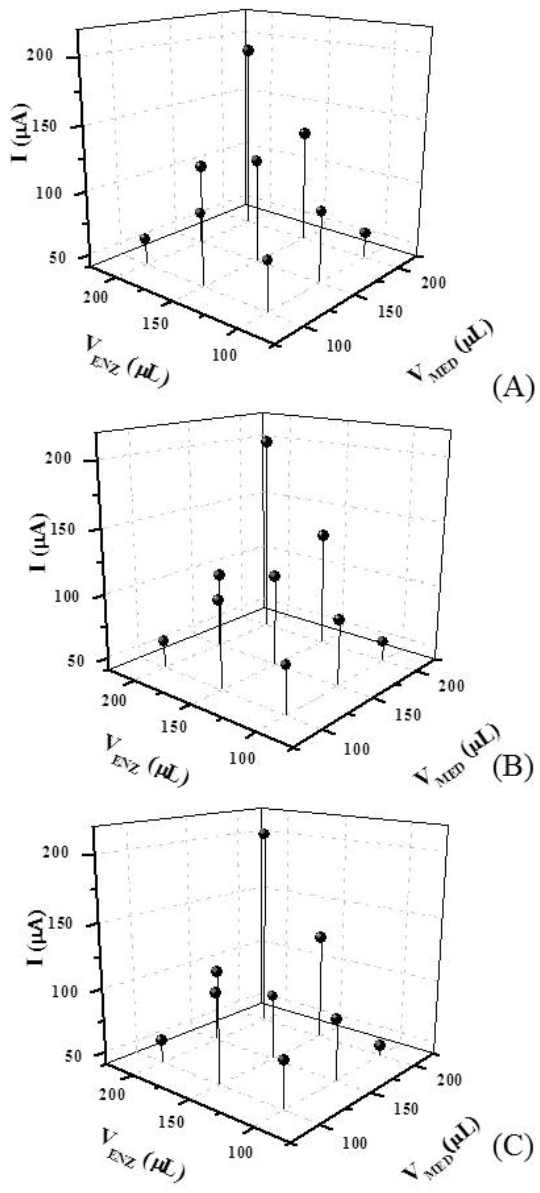

Figure 3. Dependence on the modified electrode amperometric response (I) as a function of enzyme (V_{ENZ}) and mediator (V_{MED}) amounts at different electrolyte pH: (A) 2.0; (B) 3.0; (C) 4.0. Applied potential: + 0.73 V.

The influence of pH on the amperometric response of the enzyme electrode was studied within pH range 2 - 4. The cathode current response reached a maximum value at pH 4.0, whereas electrode response diminishes below this rate. Figure 4 shows a slight variation of the amperometric response related to the pH of the combination 200 μL ABTS and 188 U mL^{-1} laccase added to the carbon paste electrodes. When the cyclic voltammogram profiles are taken into consideration, the current peak potential for the second step in ABTS oxidation, I_{pcII}, reaches the rates 187, 201 and 202 μA respectively for pH 2.0, 3.0 and 4.0.

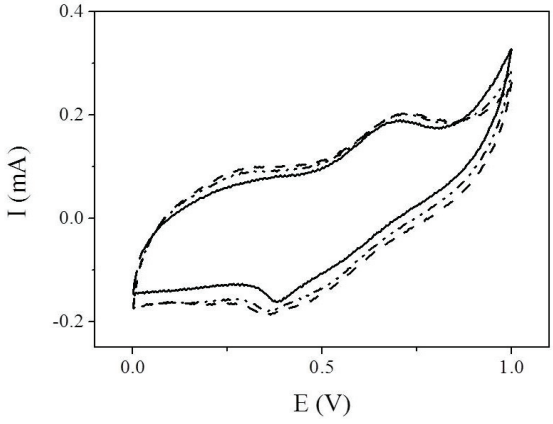

Figure 4. Cyclic voltammograms obtained for the carbon paste electrodes modified electrode with 200 μL ABTS and 188 U mL^{-1} laccase at different pH: (——) 2.0, (- .- .) 3.0 and (- - -) 4.0. Supporting electrolyte: ABTS 20 mmol L^{-1} and KCl 0.3 mol L^{-1}. s = 20 mV s^{-1}.

Conclusion

Current analysis has shown the immobilization of the enzyme laccase on carbon paste modified electrode construction. The mediator ABTS was added as carbon paste to improve the exchange mechanism at the electrode surface and extend the action of this enzyme to a variety of substrates.

The mediator was extremely effective and increased the amperometric response. Current rates (I_{pc}) had a sharp increase whilst pH 4.0 electrolyte provided better voltammetric response. The study of effect immobilized laccase concentration on the response of the electrode showed that the electrode response increased with increasing amounts of immobilized enzyme. The compositions studied for different concentrations of laccase and ABTS and pH values indicated that the concentration of laccase 200 μL ABTS and 188 U mL^{-1} laccase at pH 4.0 had the best amperometric response (202.3 μA). Current study demonstrated that modified carbon paste with laccase, with ABTS as a mediator, may be used in the construction of biosensors.

Acknowledgements

The authors would like to thank the Fundação Araucária (10015/2003; 15585/2010), NEMAN Pronex, Fundação Araucária/CNPq (17378/2009) and CNPq (478272/2007) for their financial support. Thanks are also due to NOVOZYMES Brazil for kindly providing Laccase (NOVOZYME 51003®). Author Arnaldo Fernandes da Silva Filho would like to thank IC/ProPPG/UEL for the

undergraduate scholarship. The reviewers are also thanked for their constructive criticism of an earlier version of the manuscript.

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Received on January 28, 2013.
Accepted on July 10, 2013.