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Synergic effect studies of the bi-enzymatic system laccase–peroxidase in a voltammetric biosensor for catecholamines

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Abstract

Several bi-enzymatic carbon paste biosensors modified with enzymes laccase from *Pleurotus ostreatus* fungi and peroxidase from zucchini (*Cucurbita pepo*) were constructed for evaluating the synergic effect of the two enzymes on the voltammetric biosensor response for various catecholamines. Initially was investigated the effect of pH from 5.0 to 7.5, temperature from 25 to 50 °C, initial stirring time from 30 to 150 s, scan rate from 10 to 60 mV s^{-1} and potential pulse amplitude from 10 to 60 mV on the biosensor response for several catecholamines such as dopamine, adrenaline, isoprenaline and L-dopa. It was observed a biosensor signal increase employing both enzymes, indicating thus there is a synergic effect between laccase and peroxidase, verified also in spectrophotometric studies, in the determination of these catecholamines.

Keywords: Laccase; Peroxidase; Bi-enzymatic biosensor; Synergic studies

1. Introduction

A biosensor can be defined as an analytical device that combines a biological material (e.g. enzymes, cells, microorganisms, tissues etc.) with an appropriate transducer (e.g. optic, electrochemical, calorimetric, piezoelectric etc) capable to give selective and/or quantitative analytical informations [1–4]. The biological material recognizes the analyzed substance generating a signal that is converted into a response such as current, potential, temperature variation and so on. A successful biosensor-based system must respond with application-dependent sensitivity, selectivity, reversibility, speed, and long lifetime to a desired analyte,

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while consuming minimal power and sample volume [5].

Bi-enzymatic biosensors were developed for glucose [6,7], lactate [8] and catecholamines [9] determinations employing horseradish peroxidase (HRP) and glucose oxidase; HRP and lactate oxidase, and laccase and glucose dehydrogenase, respectively. The results always show an amplification of the signal measured when these enzymes work together.

Scheller and co-workers [10] have proposed a dual enzyme amplification system based on the substrate recycling principle with respect to their applicability to catecholamine detection. In this bioelectrocatalytic approach, glassy carbon electrodes were modified by laccase or a PQQ-dependent glucose dehydrogenase. Substrate recycling occurs and the detection limit was in the nanomolar concentration range or even lower for the laccase and glucose dehydrogenase-modified electrodes. Combination of glucose dehydrogenase with laccase or tyrosinase were investigated as bi-enzymatic electrodes. Among the systems studied by those authors, the laccase/glucose dehydrogenase presented better sensitivity.

Autoxidation of some phenolics has long been known for its ability to produce superoxide and H_2O_2 [11–14]. H_2O_2 generation by the peroxidase-catalyzed oxidation of NADH is achieved by formation of a radical from NADH, and hence of superoxide, followed by dismutation of the superoxide [14]. A similar function in L-dopa, 4-methyl catechol and gallic acid were all autoxidized and generated additional H_2O_2 during *polifenol oxidase*-catalyzed oxidation, as indicated by subsequent peroxidase-catalyzed oxidation [12]. Nevertheless, the bi-enzymatic system presented in this paper (i.e. Laccase and HRP), was not found in the literature.

In the present work, enzyme *laccase*-catalyzed oxidation of catecholamine was shown to generate H_2O_2, probably via superoxide [11–14], and the H_2O_2 could then be used as an electron acceptor by *peroxidase* in a bi-enzymatic system for catecholamines determination. This system presented a synergic effect when both enzymes act together to detect substrates like cathecolamines, improving the generated signal and the analytical characteristics.

2. Experimental

2.1. Apparatus

Sorvall Products (Newtown, CN) Model Super-T21 centrifuge, provide with the model SL-50T rotor, was used in the preparation of the zucchini (*Cucurbina pepo*) crude extract.

A Hewlett-Packard (Boise, ID) Model 8452A UV–visible spectrophotometer with a quartz cell (optical path of 1.00 cm) was used in the laccase and peroxidase activity and total protein determinations.

All electrochemical experiments were carried out in a 15-ml thermostated glass cell at 25 °C. A three-electrode assembly incorporating Nujol–graphite powder modified with crude extract of laccase–peroxidase as working electrode, an Ag/AgCl (3.0 mol l^{-1} KCl) reference and platinum auxiliary electrodes were used in all measurements. Differential pulse voltammetric measurements were performed with an EG&G PAR, Model 173 A potentiostat/galvanostat connected to an EG&G PAR, Mod 175 recorder.

2.2. Reagents and solutions

All reagents were of analytical grade and all solutions were prepared with water from a Millipore (Bedford, MA) Milli-Q system (Model UV Plus Ultra-Low Organics Water). Catecholamines from Aldrich (Milwaukee, WI) 2.0×10^{-2} mol l^{-1} stock solutions were prepared daily in 0.1 mol l^{-1} phosphate buffer solution at pH 6.0. Reference solutions from 6.1×10^{-6} to 1.0×10^{-4} mol l^{-1} epinephrine, from 6.6×10^{-6} to 3.9×10^{-4} mol l^{-1} dopamine, from 6.7×10^{-6} to 7.0×10^{-5} mol l^{-1} L-dopa and from 6.2×10^{-6} to 8.1×10^{-5} mol l^{-1} isoprenaline were analyzed in a 0.1 mol l^{-1} phosphate buffer solution at the same pH. In the construction of the biosensors an Acheson 38 graphite powder from Fisher and mineral oil from Sigma were used throughout.

2.3. Crude extracts preparation

2.3.1. Laccase [15]

The microorganism *Pleurotus ostreatus* was maintained on potato-dextrose-agar PDA medium at 28 °C, and stored at 4 °C on PDA slants.

Culture medium and growth conditions: *P. ostreatus* was grown in submerged culture in Erlenmeyer flasks (1 l) containing 200 ml of Vogel [16] basal medium comprising minimal salts medium 1% m/v glucose, 2 mmol l^{-1} veratryl alcohol and 0.2% m/v yeast extract for 7 days at 28 °C, and shaken continuously at 180 rpm. Cell-free culture filtrates were obtained by centrifugation, then dialyzed against de-ionized water and freeze dried.

2.3.2. Peroxidase [17]

Twenty-five grams of the fresh peeled zucchini were homogenized in a blender with 100 ml of 0.1 mol l^{-1} phosphate buffer (pH 6.0), containing 2.5 g of Polyclar SB-100 for 2 min at 4–6 °C. The homogenate was rapidly filtered through four layers of cheesecloth and centrifuged at 13 500 rpm for 15 min at 4 °C. The resulting supernatant was stored at this temperature in a refrigerator and utilized as the enzymatic source after determinations of peroxidase activity and total protein.

2.4. Activity and total protein determinations

Laccase activity was assayed with 2,6-dimethoxyphenol (DMP) as substrate. The assay consisted in incubating a mixture containing 0.15 ml of phosphate buffer solution at pH 5.0 and enzyme (0.10 ml, diluted when necessary), in a cell of 1.00 ml-volume, at 50 °C for 5 min, and measuring the absorbance at 468 nm ($\varepsilon = 1.0 \times 10^4$ $mol^{-1}\,l\,cm^{-1}$). Laccase activity is expressed in units (U) as the number of μmol oxidized substrate $min^{-1}\,ml^{-1}$ of enzyme solution.

Peroxidase activity presented in the crude extract was determined in triplicate by measurement of the absorbance of tetraguaiacol at 470 nm produced by the reaction between 0.2 ml of supernatant solution, 2.7 ml of 0.05 mol l^{-1} guaiacol solution and 0.1 ml of 10 mmol l^{-1} hydrogen peroxide solution in 0.1 mol l^{-1} phosphate buffer solution (pH 6.5) at 25 °C. The initial rate of guaiacol peroxidation reaction was a linear function of time for 1.5–2.0 min. One activity unit is defined as the amount of enzyme that causes an increase of 0.001 absorbance per minute under the conditions described above.

Total protein concentration was determined in triplicate by the method of Hartree [18] using bovine serum albumin as standard.

2.5. Carbon paste electrodes construction

A general procedure for sensor and biosensors construction can follow three steps: firstly, mixing and homogenizing the graphite powder and the bovin serum albumin or the enzymatic crude extracts in accurate amounts during 20 min, for the sensor or biosensor construction, respectively. Secondly, homogenizing the carbon paste with mineral oil during more 20 min in a mortar. Finally, the carbon paste prepared was packed into a tip of 1-ml insulin plastic syringe and a copper wire is inserted to obtain the external electric contact. Table 1 summarizes the precise amounts of graphite, Nujol and enzymatic crude extracts used in the carbon paste biosensors preparation.

The differential pulse voltammetry (DPV) measurements were carried out varying the potential between +0.4 and −0.1 V vs. Ag/AgCl, after a suitable initial stirring time as presented in the Table 3.

Some studies to optimize the biosensor performance were evaluated such as pH effect (5.0, 6.0, 6.5, 7.0 and 7.5) in 0.1 mol l^{-1} phosphate buffer solution, initial stirring time (30, 60, 90 and 150 s), scan rate (10, 20, 30, 40, 50 and 60 mV s^{-1}), temperature effect (25, 35, 40 and 50 °C), potential pulse amplitude (10, 20, 30, 40, 50 and 60 mV) and substrates such as adrenaline, dopamine, isoprenaline and L-dopa at 1.5×10^{-3} mol l^{-1} concentration.

2.6. Spectrophotometric studies using laccase and peroxidase

Initially, the reaction of 500 μl of 60 U ml^{-1} laccase solution with 2.5 ml of 0.025 mol l^{-1}

Table 1
Carbon paste electrode and biosensor preparation

Biosensor	Graphite, (mg (%))	Nujol, (mg (%))	Enzyme, (mg (%))		Enzyme (U mg of carbon paste)	
			(Laccase)	(Peroxidase)	(Laccase)	(Peroxidase)
Blank	375.0(75)	125.0(25)	–	–	–	–
Peroxidase 1	375.0(75)	110.0(22)	–	15.0(3)		15.5
Peroxidase 2	375.0(75)	117.5(23.5)	–	7.75(1.5)		7.75
Laccase 1	375.0(75)	110.0(22)	15.0(3)	–	0.48	–
Laccase 2	375.0(75)	117.5(23.5)	7.5(1.5)	–	0.24	–
Lacc./perox.	375.0(75)	110.0(22)	7.5(1.5)	7.75(1.5)	0.24	7.75

dopamine solution was thermostated at 35 °C, and the dopaminechrome produced was spectrophotometric monitored at 464 nm at times of 5, 10 and 15 min. The same procedure was repeated for 500 μl of 88.5 U ml^{-1} peroxidase solution. To evaluate the synergic effect of both enzymes, 500 μl aliquot of 60 U ml^{-1} laccase were added to 2.5 ml of a 0.025 mol l^{-1} dopamine solution and kept at 35 °C. After 5 min, 500 μl of 88.5 U ml^{-1} peroxidase were added. The dopaminechrome obtained was spectrophotometric monitored at same experimental conditions.

3. Results and discussion

3.1. Activity and total protein determinations

As can be seen in the experimental section, spectrophotometric measures were performed to determinate the enzymatic activity, total protein and specific activity of both enzymes.

Table 2 shows the activity (U ml^{-1}) obtained using DMP and guaiacol substracts for laccase and peroxidase, respectively. These are the best substrates for each enzyme and as can be seen in this table, *P. ostreatus* fungi and zucchini (*Cucurbita pepo*) crude extracts are suitable sources of these enzymes, because they present high enzymatic activities and low total protein quantities, resulting in high specific activities (U mg of protein $^{-1}$).

3.2. Optimization of the bi-enzymatic biosensor response

To obtain the ideal experimental conditions for the carbon paste bi-enzymatic biosensor modified with crude extracts of 0.24 U of laccase (*P. ostreatus*) mg^{-1} of carbon paste and 7.75 U peroxidase (*Cucurbita pepo*) mg^{-1} of paste, some parameters such as the effect of pH, stirring initial time, scan rate, potential pulse amplitude and temperature were investigated. Table 3 summarizes the range over each variable was investigated and the optimal value found in those studies.

The effect of pH of the 0.1 mol l^{-1} phosphate buffer solution ranging from 5.0 to 7.5 on the biosensor response for 1.5×10^{-3} mol l^{-1} of each catecholamine was investigated. It was found that

Table 2
Activity, total protein and specific activity of laccase and peroxidase

Enzyme	Activity (U ml^{-1})	Total protein (mg ml^{-1})	Crude extract specific activity (U mg^{-1} of protein)	Commercial enzyme specific activity (U mg^{-1} of protein)
Laccase	60.0	3.77	15.9	10.0[a]
Peroxidase	88.5	0.171	517.0	85.0[b]

[a] (http://www.TIENZYME.com) laccase of *P. ostreatus*.
[b] (http://www.worthington-biochem.com/priceList/P/HPO.html) peroxidase (crude).

Table 3
DPV parameters studied and optimized for the bi-enzymatic electrode containing 0.24 laccase U mg^{-1} carbon paste and 7.75 peroxidase U mg^{-1} carbon paste

Analyte	pH	Stirring time (s)	Scan rate (m Vs^{-1})	Potential pulse amplitude (mV)	Temperature (°C)
Range studied	6.0–7.5	30–150	10–60	10–60	25–50
Dopamine	6.0	60	40	50	35
Adrenaline	7.0	60	40	50	35
L-dopa	6.5	60	40	50	35
Isoprenaline	6.0	60	40	50	35

pH values of 6.0, 7.0, 6.5 and 6.0 for dopamine, adrenaline, L-dopa and isoprenaline, respectively, were the optimum for these catecholamines.

The effect of initial stirring time in an interval from 30 to 150 s on the biosensor response for 1.5×10^{-3} mol l^{-1} of each catecholamine was investigated to determine the best equilibrium time for the reaction. The analytical signal (cathodic current) increased with the initial stirring time until 60 s and it did not considerably change at times between 61 and 150 s. Therefore, an initial stirring time of 60 s was selected in further experiments. In addition, the lifetime of this biosensor was about 2 months ($\sim$ 500 determinations with the same carbon paste containing both enzymes).

The effect of the potential scan rate between 10 and 60 mV s^{-1} on the electrode response for 1.5×10^{-3} mol l^{-1} of each catecholamine solution was investigated. The optimum cathodic peak current obtained for differential pulse voltammetry (DPV) measurement was obtained at a scan rate of 40 mV s^{-1}. The DPV was chosen due to improve the analytical signal, however, it does not interfere in the determining step, which is the enzymatic reaction.

As each enzyme presents a best temperature condition, the temperature effect from 25 to 50 °C on the biosensor response for 1.5×10^{-3} mol l^{-1} for each catecholamine was investigated. The employment of 35 °C resulted in a higher analytical signal and was selected for further studies with the bi-enzymatic system.

3.3. Synergic effect evaluation

Fig. 1 shows a possible mechanism of laccase–peroxidase oxidation of these catecholamines by hydrogen peroxide generation. Initially, before the biosensor polarization and under 100 rpm for 60 s, a generic catecholamine can be oxidized by laccase enzyme in the molecular oxygen presence generating the correspondent aminequinone (a) and hydrogen peroxide via superoxide ($O_2^{\bullet}$) [11,12]. Simultaneously, the H_2O_2 produced at the biosensor surface can be used as an electron acceptor by peroxidase in the oxidizing process of other catecholamines not yet oxidized by laccase to quinones (b). After the biosensor polarization, the catecholaminequinones produced in both enzymatic process were reducing back to the original catecholamines and as the result of this synergic process, higher cathodic current peaks were obtained when laccase and peroxidase act together in the same biosensor (a and b). The signal was 29.6% in average higher than those obtained for each biosensor individually containing the same proportion of enzymatic units concluding that there is a synergetic effect between these enzymes when immobilized in the same biosensor.

Fig. 2 shows the differential pulse voltammograms obtained In 0.1 mol l^{-1} phosphate buffer solution at pH 6.0 of 2.3×10^{-3} mol l^{-1} dopamine, as example, using a carbon paste (blank) electrode (a), a 15.5 U peroxidase mg^{-1} of paste (b), a 0.48 U laccase mg^{-1} of paste (c) and the bi-enzymatic 7.75 U peroxidase and 0.24 U laccase mg^{-1} of paste biosensor (d). As can be seen, the

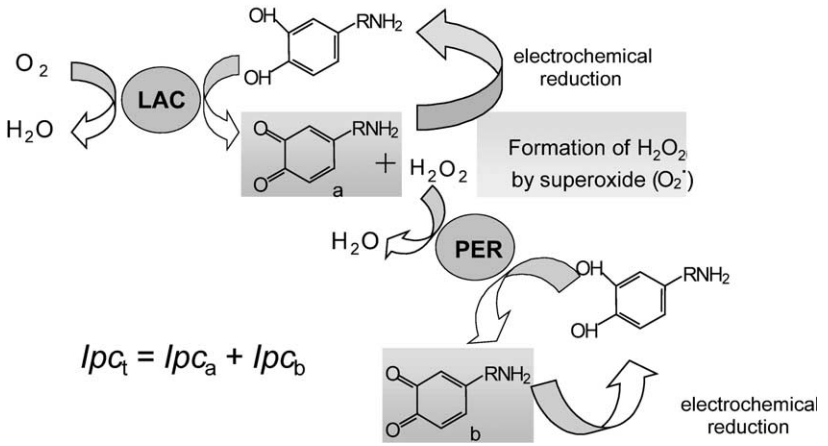

Fig. 1. Schematic mechanism of catecholamines oxidation by the laccase–peroxidase system.

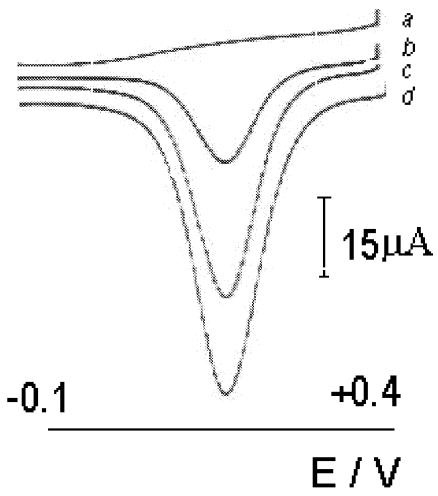

Fig. 2. Differential pulse voltammetric studies using (a) blank (plain carbon paste electrode); (b) peroxidase (15.5 U mg^{-1} paste); (c) laccase (0.48 U mg^{-1} paste); and (d) laccase–peroxidase (0.24 and 7.75 U mg^{-1} paste) biosensors in a stirring time of 60 s, scan rate of 40 mV s^{-1}, potential pulse amplitude 50 mV in 0.1 mol l^{-1} phosphate buffer solution (pH 6.0) for 2.3×10^{-3} mol l^{-1} dopamine solution.

later showed higher current peaks, due to synergic effect.

Fig. 3 presents the i_{pc} obtained for determination of 1.5×10^{-3} mol l^{-1} adrenaline, dopamine, isoprenaline and L-dopa solutions, respectively using the biosensors developed under pre-selected optimized conditions (i.e. phosphate buffer in an appropriate pH, 40 mV s^{-1} of scan rate, 60 s of stirring initial time, at 35 °C) by differential pulse voltammetry. The synergic effect of 0.24 U laccase mg^{-1} of paste and 7.75 peroxidase U mg^{-1} of paste bi-enzymatic biosensor for those solutions was than evaluated. It was concluded that the bi-enzymatic biosensor presents the better response related to the others, once the response of 7.75 U peroxidase mg^{-1} of paste and 0.24 U laccase mg^{-1} of paste biosensors separately showed a lower final current sum than the final current generated by the proposed bi-enzymatic biosensor.

As can be seen in the Fig. 2, the presence of both enzymes in the bi-enzymatic electrode resulted in a synergic effect in the oxidation of catecholamines (e.g., dopamine), producing higher cathodic peaks.

3.4. Spectrophotometric studies of the bi-enzymatic system

Kinetic studies of the reaction involving 0.025 mol l^{-1} dopamine solution with 10 U ml^{-1} laccase and 14.8 U ml^{-1} peroxidase were developed to confirm the results obtained in the electroanalytical studies. Fig. 4 shows the absorbance results vs. reaction time involving each catecholamine with the enzymes individually and the bi-enzymatic system proposed. The generation of hydrogen peroxide catalyzed by laccase enzyme in this reaction permits the dopamine oxidation by per-

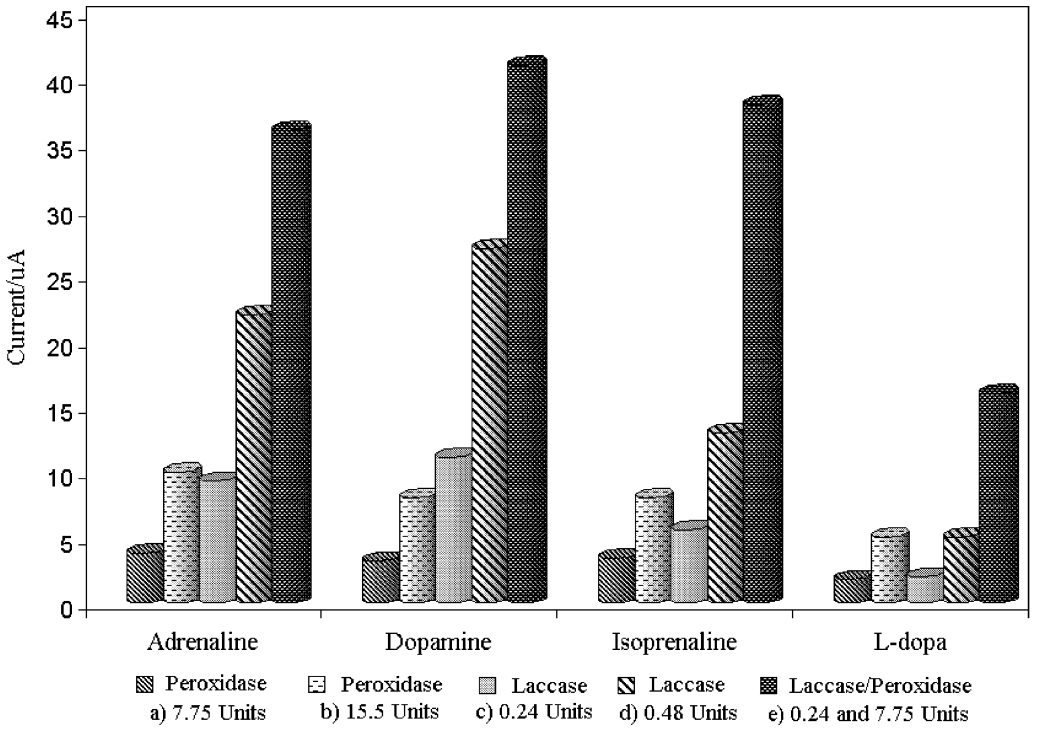

Fig. 3. Catecholamines determination using (a) peroxidase (7.75 U mg^{-1} paste); (b) peroxidase (15.5 U mg^{-1} paste); (c) laccase (0.24 U mg^{-1} paste); (d) laccase (0.48 U mg^{-1} paste) and (e) laccase–peroxidase (0.24 and 7.75 U mg^{-1} paste) biosensors at stirring time of 60 s, scan rate of 40 mV s^{-1} and potential pulse amplitude 50 mV in 0.1 mol l^{-1} phosphate buffer solution (pH 6.0), for 2.3×10^{-3} mol l^{-1} of each catecholamine (adrenaline, dopamine, isoprenaline and L-dopa) 2.3×10^{-3} mol l^{-1} dopamine; 2.3×10^{-3} mol l^{-1} isoprenaline and 2.3×10^{-3} mol l^{-1} L-dopa solutions, respectively.

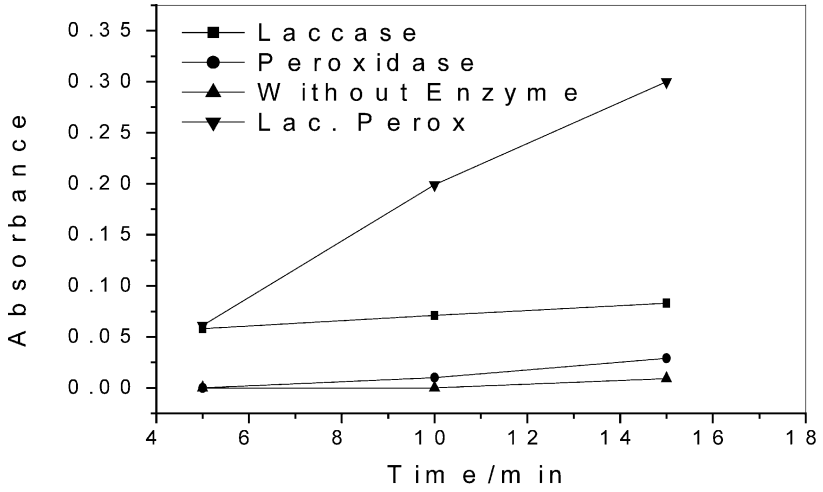

Fig. 4. Spectrophometric kinetic studies involving *Laccase* (■), *Peroxidase* (●), the *Laccase-Peroxidase systems* (▼) and without enzyme (▲) for 0.025 mol l^{-1} dopamine solution.

oxidase, obtaining an analytical signal 70% higher than that analytical signal obtained by both enzymes individually in the same experimental conditions. The absence of hydrogen peroxide in the peroxidase reaction with the catecholamine confirms that there is no increasing of absorbance in this case. These studies were carried out for the other catecholamines and the results were very similar, concluding that there is a synergetic effect between these enzymes in the catecholamine oxidation process.

3.5. Analytical figures of merit

After the optimization of the working conditions of the bi-enzymatic electrode containing 0.24 U laccase mg^{-1} of paste and 7.75 peroxidase mg^{-1} of paste, analytical curves using differential pulse voltammetric measures were built to evaluate the sensitivity and linearity for each catecholamine studied. It was observed linearities from 6.6×10^{-6} to 3.9×10^{-4}, from 6.1×10^{-6} to 1.0×10^{-4}, from 6.7×10^{-6} to 7.0×10^{-5} and from 6.2×10^{-6} to 8.1×10^{-5} mol l^{-1}, for dopamine, adrenaline, L-dopa and isoprenaline solutions, respectively. The detection limits observed were 2.7×10^{-8}, 2.5×10^{-8}, 2.4×10^{-8}, 2.6×10^{-8} mol l^{-1}, for dopamine, adrenaline, L-dopa and isoprenaline, respectively.

4. Conclusions

As can be observed, both enzymes together showed a synergetic effect in the catecholamines oxidation reaction, producing higher DPV cathodic peaks ($\sim$30% higher). In addition, the analytical signal (absorbance) was 70% higher than the sum of each analytical signal obtained individually, validating the hypothesis of synergetic effect between both enzymes.

The probable mechanism is by hydrogen peroxide production, via superoxide, in the laccase oxidation system. This peroxide is used then by peroxidase to improve the oxidation of the same catecholamine, as can be observed in other similar systems [11–14].

In conclusion, the bi-enzymatic electrode developed was successfully used to show the synergic effect in the catecholamines determination.

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References

[1] I.C. Vieira, O. Fatibello-Filho, Anal. Lett. 30 (1997) 895.
[2] O. Fatibello-Filho, I.C. Vieira, Fresen. J. Anal. Chem. 368 (2000) 338.
[3] O. Fatibello-Filho, I.C. Vieira, J. Braz. Chem. Soc. 11 (2000) 412.
[4] O. Fatibello-Filho, M.D. Capelato, Quim. Nova 15 (1992) 28.
[5] A.J. Ricco, R.M. Crooks, Acc. Chem. Res. 31 (1998) 200.
[6] M.A. Del Cerro, G. Cayuela, A.J. Reviejo, J.M. Pingarron, J. Wang, Electroanalysis 9 (1997) 1113.
[7] E. Corton, F. Battaglini, J. Electroanal. Chem. 511 (2001) 8.
[8] J. Kulys, A. Drungiliene, U. Wollenberger, K. Krikstopaitis, F. Scheller, Electroanalysis 9 (1997) 213.
[9] L. CocheGuerente, S. Cosnier, L. Labbe, Chem. Mater. 9 (1997) 1348.
[10] F. Lisdat, U. Wollenberger, A. Makower, H. Hortnagl, D. Pfeiffer, F.W. Scheller, Biosensors Bioelectron. 12 (1997) 1199.
[11] J. Michalowski, P. Halaburda, Talanta 55 (2001) 1165.
[12] Y. Jiang, P.W. Milies, Phytochemistry 33 (1993) 29.
[13] F.P. Misra, I. Fridovich, J. Biol. Chem. 247 (1972) 3170.
[14] G. Cohen, R.E. Heikkila, J. Biol. Chem. 249 (1974) 2447.
[15] O.D. Leite, A.S. Endo, M.P. Felisbino, S.M. Obara-Doi, R.C. Fonseca, A.M. Barbosa, Third Latin American Biodegradation and Biodeterioration Symposium, Book of Abstracts, Florianópolis, Brazil, September, 34 (1998).
[16] H. Vogel, J. Genet. Bull. 13 (1956) 42.
[17] I.C. Vieira, O. Fatibello-Filho, L. Angnes, Anal. Chim. Acta 398 (1999) 145.
[18] E.S. Hartree, Anal. Biochem. 48 (1972) 422.