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LACCASE POLYMERIZATION OF AMINO-PHENOL COMPOUNDS

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Introduction

Laccases are a multi-copper phenol oxidase, which reduces oxygen to water and simultaneously catalyze the oxidation of aromatic pollutant such anilines and phenols [1, 2]. Several methods using laccase, immobilized laccase and laccase/mediator system have been developed for the treatment of the textile effluents [3-11]. This enzyme decolorizes some azo dyes without direct cleavage of the azo bond through a highly non-specific free radical mechanism, thereby avoiding the formation of toxic aromatic amines [12]. However, the substrate specificity of laccase limits the number of azo dyes that can be degraded [13]. Another possibility is the use of an azo reductase bacteria, that under microaerophilic condition can cleave a wider range of azo dyes into corresponding toxics aromatic amine [12, 14, 15], associated with a laccase [16].The microaerophilic condition has permitted the simultaneously presence, in the medium, of the azo reductase and a *Trametes villosa* laccase that is able to polymerize various substituted anilines [17]. Usually these types of reactions perform an oxidative oligomerization established by a nonenzymatic coupling reaction between substituted anilines. However in order to enhance the degree of polymerization, the catechol, a diphenolic compound, was added to the effluent. The catechol is a known substrate for laccase that polymerize forming a poorly soluble product [18]. The presence of catechol disfavors the aromatic amine self-coupling and enhances the coupling between catechol and the amines [19]. The copolymerization, between the oxidized anilines and catechol in the effluent, performed by simultaneously nonenzymatic coupling and enzymatic polymerization showed products with low solubility. These kinds of reaction products were not observed when the anilines and catechol separately were reacted in presence of laccase in the same conditions. The formation of the insoluble products brings the advantage that they can be removed from effluent in the form of a precipitate by further treatment processes. This reaction has been compared on the basis of the kinetic parameters using a HBT/laccase system. HBT (1-hydroxybenzotriazole), the most researched mediator that is able to oxidize a variety of aromatic compounds, was used to broaden the range of azo dyes and to increase the polymerization rate of laccase [20].

Experimental

Chemicals. 1-hydroxybenzotriazole, 2,5-diaminobenzene sulfonic acid and the salts were purchased from Sigma, St. Louis, MO. All chemicals were of high purity and used as received. Laccase (EC 1.10.3.2) from *Trametes villosa* (5.3 mg protein/mL, 600 U/mL) was kindly provided by Novo Nordisk, Denmark.

Electrode preparation. For the experiments was used as working electrode a glassy carbon electrode. Prior to the experiments the surface of the glassy carbon electrode was successively polished with 5, 1, 0.3 and 0.05 µm alumina polish (Buehler Ltd, USA) and then rinsed with 8 M nitric acid and distilled water before use.

Electrochemical experiments. All the electrochemical experiments were performed using a Voltalab 30 Potentiostat (Radiometer Analytical, France), controlled by the Voltamaster 4 (version 5.6) electrochemical software. The working, counter and reference electrodes were respectively: glassy carbon electrode (0.07 cm^2), coiled platinum wire (23 cm) and an Ag|AgCl electrode filled with 3M NaCl (BAS, Bioanalytical Systems, West Lafayette, IN, USA). The supporting electrolyte used in the electrochemical cell was a solution of 0.1 M sodium-citrate buffer pH 5. All solutions were deoxygenated through bubbling nitrogen for 20 min before measurements. All experiments were performed in bulk using amperometric detection. The applied potential was -50 mV *vs.* Ag|AgCl.

Coupling experiments. Equimolar solutions of 2,5-diamino benzene sulfonic acid (DBSA) and catechol (10 mM; total volume 2.5 mL) buffered with 0.1 M sodium citrate buffer, pH 5, were incubated with 20 µL of laccase and 0.5 mL of buffer in a standard stirred cuvette at 25ºC. In the case of experiments with mediator the buffer volume (0.5 mL) was replaced by 10 mM buffered solution of 1-hydroxybenzotriazole (HBT). Another experiment was performed with a 2,5-diamino benzene sulfonic acid and laccase premixed solution and the catechol was added successively. The same experiments were performed with catechol and 2,5-diamino benzene sulfonic acid separately.

Results and Discussion

Laccase shows broad specificity in the process of oxidizing many compounds (mainly of phenolic type) and can be exploited for the oxidation/biodegradation of a number of aquatic and terrestrial xenobiotics, industrial wastewaters, as well as for biotechnological treatment of industrial products [21]. Laccase catalyzes the oxidation of organic substrates such as phenolic compounds by molecular oxygen in homogeneous solutions. In the presence of soluble electron donors, laccase can be reduced in a mediated electron transfer (MET) mechanism. In this mechanism the electron donor (substrate) penetrates the active site of the enzyme where it is oxidized in a single electron oxidation step often producing an electrochemically active compound (possibly a radical) that in turn can be re-reduced at the electrode surface in a mediated electron transfer (MET) step. Initial experiment (applied potential -50mV versus Ag|AgCl) showed that the electrode has low noise/background current while upon the injection of the solution of the azo dyes they generate a reduction current. Such a response is relatively well understood and it is usually ascribed to electrochemical reduction of laccase oxidation products [22]. The responses are dependent on the concentration of the substrate in the solution of interest. At higher substrate concentrations the current-concentration dependence gradually reached saturation. The apparent Michaelis–Menten constants (K_m^{app}) and maximal currents (I_{max}) have been calculated by fitting the variation of current–concentration dependencies of the analyzed compounds to the electrochemical Michaelis–Menten equation [23]. K_m^{app} is an indicator of the affinity that an enzyme has for a given substrate, and hence the stability of the enzyme-substrate complex.

$$I = \frac{I_{max}[S]}{[S] + K_m^{app}} \qquad (1)$$

where S is the substrate concentration, I_{max} the maximum current and K_m^{app} the apparent Michaelis–Menten constant. The calculated values of K_m^{app} (calculated from Hanes–Woolf linearization of the equation (1)) and the catalytic efficiencies are presented in Table 1. The kinetics of mediated laccase catalyzed reactions is firstly affected by the affinity between enzyme and the mediator. An estimation of this influence can be done by amperometric measurements in terms of I_{max}/K_m^{app} ratio. These parameters are often calculated in the design of enzymatic sensors to evaluate the sensitivity of the system proposed, which is related to the low or high affinity of the enzyme towards a specific substrate. Lower K_m^{app} values at similar catalytic currents involved higher effectiveness of the enzyme at lower mediator concentrations. The feasibility of oxidative coupling between xenobiotics in the presence of oxidoreductive enzymes for the remediation of environmental pollution has been described by various researchers [24]. However, little of the harmful chemicals from the nature can be transformed by the enzyme alone. It has been shown that adding a second, easily oxidizable molecule can substantially enhance the reactivity of the inert substance through the formation of a free radical, reacting with the recalcitrant xenobiotic. In the second stage, the oxidation products are subject to chemical coupling. If the reaction takes place in polluted aqueous environments, the oxidation products couple primarily to each other and precipitate out of solution in the form of non-toxic polymers [25, 26]. Thereby, in our studies we used catechol as precursor to enhance the possibility of removal of the aromatic amines formed during the azo dye degradation. The product formed during the coupling reaction might be a polymer which can be easily removed from the environment through physical techniques. Initially, there were performed experiments to check the if there is any response for the 2,5-diamino benzene sulfonic acid (DBSA) in presence of catechol or in the presence of laccase. Addition of catechol or of the laccase to the system gave no change in the current even if HBT (as mediator) was added to the solution. The absence of a measurable signal at the used concentration of DBSA permitted us to run further experiments in order to study the unmediated and mediated coupling of the catechol with DBSA in presence of laccase. Firstly the response of the catechol oxidation by laccase was monitored in the absence and in the

presence of 100 μM HBT. Since the response of the sensor is proportional to the concentration of the catechol in solution, then if the catechol is consumed in the coupling reaction with DBSA this will be observed as decay in the current. When the coupling reaction was studied in the absence of a mediator it could be observed that the response of the electrode for the same amount of catechol added to the electrolyte solution was significantly diminished in the presence of DBSA or if the catechol was added after previous mixing with DBSA (equimolar ratio) than in the case when the current measured was due to the oxidation of catechol by laccase (figure not shown). As can be seen from Table 1, for this case the values of I_{max} is decreased in both cases when DBSA is present in the electrolyte solution, and, moreover an increase in the values of K_m^{app} is observed leading us to the conclusion that a competitive reaction (coupling of the catechol with DBSA) might take.

Table 1. Results obtained for the coupling reaction of the DBSA with catechol

	I_{max} (μA)	K_m^{app} (μM)	I_{max}/K_m^{app} ($\mu A*\mu M^{-1}$)
Catechol and laccase	2.40	147.00	0.02
DBSA and laccase	NA	NA	NA
DBSA and catechol	NA	NA	NA
DBSA/laccase premixed and catechol	1.70	174.80	0.01
DBSA/catechol premixed and laccase	1.90	226.00	0.01
Catechol and laccase + HBT	1.50	261.00	0.01
DBSA and laccase + HBT	NA	NA	NA
DBSA and catechol+ HBT	NA	NA	NA
DBSA/laccase premixed and catechol + HBT	3.10	133.50	0.02
DBSA/catechol premixed and laccase + HBT	1.10	118.00	0.01

In the presence of HBT as mediator it was also observed a diminution in the current registered for the case when the catechol and DBSA were mixed (equimolecular ratio) prior to the addition to the electrolyte solution (marked with • in Figure 1) than in the case when the catechol addition was made just in the presence of laccase and of the HBT (marked with ■ in Figure 1). Surprisingly when the addition of the catechol to the system was made after addition of the HBT and of the DBSA to the system it was observed that the registered currents for catechol (marked with ▲in Figure 1) are much higher than in the absence of DBSA (amplification factor of 2). It might be assumed that in the premixed solution of DBSA and catechol is formed coupling product and that the presences of catechol unfavoured the aromatic amine self-coupling [19, 27]. But this behavior is not fully understood yet and is going to make the object of our future studies. Beside, from the results presented in Table 1 it can be seen that the values obtained for the K_m^{app} are increasing when the reaction occurs in presence of DBSA compared with the case when in absence of it, results that are in contrast with what it was observed when the mediator (HBT) was not present in the system. However, as a full understanding of the interaction between catechol and DBSA in the absence and in the presence of HBT and its implications on the Michaelis-Menten remain to be elucidated, it is here only possible to give the experimental results without a thorough explanation to what is obtained.

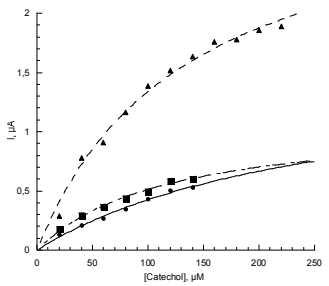

Figure 1. Calibration graph obtained for oxidation of catechol by laccase in presence of HBT. • -catechol premixed with DBSA, ■- catechol alone, ▲- catechol added after prevoius addition of DBSA to the system, in 0,1 M citrate buffer pH 5.0, at -50mV vs. Ag|AgCl electrode filled with 3M NaCl.

Conclusions

The present study reveals the possibility of removal of the aromatic amines eventually obtained in the degradation of the azo dyes processes.

In our opinion the main advantages of this coupling of the formed aromatic amine with the catechol is brought by the fact that if this method is used for removal of the aromatic amines from the polluted water or soil then is not necessary an further addition of the catechol to the system, since is already existent in the humic substances of the soil.

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