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Electrochemistry and kinetics of fungal laccase mediators

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Abstract

The screening of potential redox mediators for laccase was performed using homogeneous *Trametes hirsuta* laccase. Heterogeneous (electrochemical) and homogeneous (oxidation by laccase) reactions of the different types of the enhancers (mediators) of the enzyme were investigated. It was discovered that derivatives of phenyl-methyl-pyrazolones and benzoic acid, as well as *N*-hydroxynaphthalimide were efficient substrates for the laccase. The characterization of several representatives from each class was carried out using electrochemical and enzyme kinetics methods. The kinetic parameters for the oxidation of phenyl-methyl-pyrazolones and 3-(6-hylroxy)-aminobenzoic acid were comparable to those for 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) oxidation by the laccase, whereas the rate of enzymatic oxidation of *N*-hydroxynaphthalimide was sufficiently lower. Electrochemical experiments demonstrated that only oxidation of phenyl-methyl-pyrazolones and *N*-hydroxynaphthalimide yielded several high-potential intermediates capable of oxidizing veratryl alcohol, which was used as a lignin model substrate, whereas derivatives of benzoic acid showed low-potential intermediate, which was not able to oxidized lignin model compound. Phenyl-methyl-pyrazolones was about 50% as effective in degrading veratryl alcohol compared to ABTS as judged from HPLC kinetic studies, whereas *N*-hydroxynaphthalimide showed the same efficiency as ABTS. Phenyl-methyl-pyrazolones and hydroxynaphthalimides may be of commercial interest for oxidoreductase-catalyzed biodegradation of different xenobiotics.

Keywords: Laccase; Mediator; Enhancer; Phenyl-methyl-pyrazolone; Benzoic acid; Hydroxynaphthalimide

1. Introduction

Laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) catalyzes oxidation of ortho- and para-diphenols, aminophenols, polyphenols, polyamines, lignins and aryl diamine as well as some inorganic ions, coupled to the reduction of molecular dioxygen to water [1,2]. The enzymes belong to a group of "blue" multicopper oxidases and they are classified into two groups in accordance with their source, i.e. plant and fungal. However, diphenol oxidases (laccases) have also been identified in eubacteria [3–5] and insects [6,7]. Laccase contains four metal ions historically classified into three types, e.g. T1, T2, T3, according to their spectral characteristics [2].

The key characteristic of laccase is the standard redox potential of the T1 Cu site. The value of the redox potential of the T1 site has been determined using potentiometric titrations with redox mediators for a great number of different laccases and varies between 430 and 790 mV vs. NHE [8–14]. It has been shown for some laccases that the T1 site is the primary center accepting electrons from donor substrates [1,2,9] or electrodes [14,15]. In addition, the catalytic efficiency (k_{cat}/K_M) for some donor substrates depends on the redox potential of the T1 site [9,16]. This observation explains why laccases

Abbreviations: HQ, hydroquinone; PP, 1-phenyl-3-methyl-pyrazolone-5; SPP-m, 1-(3′-sulfophenyl)-3-methyl-pyrazolone-5; SPP-p, 1-(4′-sulfophenyl)-3-methyl-pyrazolone-5; PPA, 1-phenyl-2,3-methyl-4-amino-pyrazolone-5; PPA-Na, sodium 1-phenyl-3-methyl-4-methylamino-pyrazolone-5-N(4)-methanesulfonate; *N*-(OH)PhI, *N*-hydroxyphthalimide; *N*-OH-NaphI, 1,8-*N*-hydroxynaphthalimide; HABA, 3-(6-hylroxy)-aminobenzoic acid; ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate); VOH, veratryl alcohol (3,4-dimethoxybenzyl alcohol); VCOH, veratryl aldehyde (3,4-dimethoxybenzylaldehyde).

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with high redox potentials of the T1 sites are of special interest in biotechnology, particularly for different bleaching [17–19] and bioremediation processes [20,21].

Laccase is a mandatory enzyme for lignin conversion, since fungal laccase-deficient mutants completely loose the ability to degrade lignin [22,23]. However, the mechanism of lignin transformation in nature is very complex, and the role of laccase in this process is not fully understood. Lignin, a highly branched, irregular three-dimensional organic polymer, is the most abundant biopolymer in Nature next to cellulose [24]. This natural polymer contains different structures including phenolic and non-phenolic compounds. Conventionally, the role of fungal laccases in lignin degradation was thought to be limited only to the oxidation of low-redox potential phenolic substructures of the polymer. This conclusion was based on the values of the redox potentials of T1 sites of the enzymes, which do not exceed 800 mV. When natural and artificial redox mediators (better referred as "enhancers") of the enzyme were found, it was realized that laccase can play a broader role in lignin degradation [25–27]. Mediators or enhancers of laccase are enzyme substrates, which, being enzymatically oxidized, form highly reactive intermediates [28–30]. These intermediates can react with non-phenolic, strong lignin substructures, resulting in degradation of wood [26,31–33]. The major difference between a mediator and an enhancer is the ability of the first one to cycle between its oxidized and reduced states for unlimited period of time. True redox mediators are not consumed during the redox transformation, whereas enhancers fall out from the catalytic cycle of the enzyme [34–36]. To a first approximation, only complexes of transition metals can be true mediators of laccase, whereas all organic substrates are essentially enzyme "enhancers" [35,36]. However, the term "mediator" has been conventionally used for all enhancers of laccases. The confusion in the terminology is still unresolved. Therefore, although both terms are used in this work with respect to the studied substances, we actually mean the "enhancer" nature of the substrates.

Laccase-mediator (or laccase-enhancer) systems are employed in different biotechnological processes, such as green biodegradation of xenobiotics including pulp bleaching [17,26], bioremediation [21], labeling in immunoassays [37], and green organic synthesis [38]. However, broader application of well-known mediators is restricted by their high price and limited effectiveness. One of the prospective trends in laccase research is screening and identification of new, low-cost redox mediators of laccases. The studies of new mediators are aimed at understanding the mechanism of their redox transformations, their efficiency in homogeneous reactions catalyzed by laccase, and, finally, their ability to degrade different kinds of xenobiotics.

Electrochemical studies of different laccase-mediator systems were performed previously [39–43]. We have shown in our recent publications that phenyl-methyl-pyrazolones are promising enhancers for laccase in the enzymatic degradation of lignin and its model compounds [36,44]. Low-cost enhancers can broaden the application of laccase-mediator systems in large-scale biotechnological processes. In the present study, the effect of different substitutions into the structure of phenyl-3-methyl-pyrazolones on the efficiency of degradation of lignin model compounds has been investigated. The novel enhancers have been compared to the substances from other structural groups, namely, derivatives of benzoic acid and *N*-hydroxynaphthalimides, under the same experimental conditions.

2. Materials and methods

2.1. Chemicals

1-Phenyl-3methyl-pyrazolone-5; 1-phenyl-2,3-di-methyl-4-amino-pyrazolone-5; *N*-hydroxyphthalimide; 1,8 *N*-hydroxynaphthalimide; 3-amino(6-hylroxy)-benzoic acid; 3-chloro(6-hylroxy)-benzoic acid; 6-(3-chloro)-aminobenzoic acid; and 4-(*N*-urea)benzoic acid were synthesized in the Institute of Phytopathology (Moscow, Russia). 1-(3′-Sulfophenyl)-3-methyl-pyrazolone-5 and 1-(4′-sulfophenyl)-3methyl-pyrazolone-5 have been kindly provided by Prof. Ir Gvon Khan (Federal State Unitary Enterprise "The State Scientific NIOPIK"). Sodium 1-phenyl-3-methyl-4-methylamino-pyrazolone-5-N(4)-methanesulfonate was from PharmaMed Naturals (Moscow, Russia). 3,4-Dimethoxybenzyl alcohol, 3,4-dimethoxybenzaldehyde, and 3,4-dimethoxybenzoic acid were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Na_2HPO_4, $C_6H_8O_7$, KOH, and H_3PO_4 were of the highest purity available from Russian sources. 0.1 M citrate-phosphate buffer, pH 5.0, was made by mixing solutions containing 0.1 M citric acid and 0.1 M Na_2HPO_4. Poor soluble compounds were preliminary dissolved in distilled ethanol.

2.2. Enzyme

Basidiomycete *Trametes hirsuta* (Wulfen) Pilát was obtained from the laboratory collection of the State Research Institute of Protein Biosynthesis (Moscow, Russia). Accounts of the production of laccase on a preparative scale and characterization of the enzyme in detail have been previously published [12]. Homogeneous preparation of laccase was stored in 0.1 M phosphate buffer, pH 6.5, at −18 °C. The specific activity of the enzyme was ca. 140 units/mg of proteins. The activity of laccase was determined spectrophotometrically in 0.1 M citrate-phosphate buffer, pH 4.2, containing 10 mM catechol as a substrate using a Hitachi-557 spectrophotometer (Tokyo, Japan) as described in [12]. One unit of activity is defined as the amount of laccase oxidizing 1 μmol of catechol per min under standard conditions. Specific activity is expressed in units of activity per mg of protein.

2.3. Steady-state kinetics

Determination of rate constants was performed using the data on oxygen consumption with a Clark type electrode and a voltammetric analyzer CV-50W BAS (Bioanalytical Systems, West Lafayette, IN, USA) in accordance with the method described in [45]. A substrate solution in 0.1 M citrate-

phosphate buffer, pH 5.0 (1.0 mL), was placed into an electrochemical cell, and the reaction was initiated by the addition of laccase. The initial oxygen concentration was taken equal to 260 μM. All measurements were performed in triplicate at 20 °C. Kinetic parameters were calculated from the Michaelis–Menten equation using a Microcal Origin version 5.0 programme. The enzymatic reactions for poor soluble organic substances were performed in 0.1 M citrate-phosphate buffer, pH 5.0, containing 2% ethanol. Noteworthy, control experiments were performed for all mixtures used and necessary corrections were done. For instance, it was taken into account that the presence of 2% ethanol in the buffer solution decreased the activity of laccase by 5% and increased the solubility of molecular oxygen by 4%.

2.4. Cyclic voltammetry

A glassy carbon electrode (GC, 4.3 mm diameter, Russia) served as working electrodes. For each experimental series with one enhancer, the electrode surface was carefully polished on alumina FF slurry (0.1 μm, Stuers, Copenhagen, Denmark), rinsed with double-distilled water, sonicated after polishing for 10 min, then rinsed thoroughly with double-distilled water and allowed to dry. Cyclic voltammograms (CVs) were recorded in a three-electrode electrochemical cell (sweep scan rates, 1–1000 mV/s; potential range, 0–1150 mV) using a potentiostat BAS CV-50W Electrochemical Analyzer controlled with the software v. 2.1 BAS CV-50W. In these experiments, an $Hg|Hg_2Cl_2|KCl_{sat}$ electrode was served as a counter electrode, and an $Ag|AgCl|KCl_{sat}$ reference electrode (200 mV vs. NHE) was used. CVs were recorded in 0.1 M citrate-phosphate buffer, pH 5.0, in the absence or presence of 0.2 mM enhancer. In the case of *N*-(OH)NaphI, 2% ethanol was added into the buffer. Before and after recording CVs, the surface of the working electrode was refreshed by polishing with PK-4 (BAS) polishing kit. All values in the figures and in the text are expressed as potentials against the $Ag|AgCl|KCl_{sat}$ electrode (200 mV vs. NHE).

2.5. HPLC studies

Analysis of veratryl alcohol oxidation products was performed by reverse phase hydrophobic HPLC on a Luna C18 column (250 × 4.6 mm) (Phenomenex, Torrance, CA USA) with a linear gradient of acetonitrile (5–95%) in 0.086% H_3PO_4 at a 1 mL/min flow rate using a Stayer-system (Acvilon, Moscow, Russia). The incubation mixture contained 10^{-3} M veratryl alcohol, $5 \cdot 10^{-3}$ M enhancer, and 10^{-6} M *T. hirsuta* laccase in 0.1 M citrate-phosphate buffer, pH 5.0, at 20 °C. In the control mixture either laccase or mediator was omitted. The sample preparation included ultrafiltration through a PM-10 membrane in an Amicon cell (Billerica, MA, USA) to remove all proteins, followed by acidification of the filtrate with 0.086% H_3PO_4 (v/v). Products were identified in accordance with their retention times. Veratryl alcohol, veratryl aldehyde, and veratric acid were used as standards. Quantification of the products in the eluate was performed by integrating the elution peaks using Multichrom software (BioChimMak, Moscow, Russia).

3. Results and discussion

3.1. Rational for screening redox enhancers

The reactivity of aromatic and heterocyclic compounds is known to be dependent on the substituent nature and its position. The effect of substituents on the oxidation of these compounds is determined by both electronic and spatial factors. The goal of the work was to select laccase enhancer compounds with the best kinetic parameters in homogeneous reaction and optimal parameters (potential, reversibility) for the electrochemical reaction on the electrode. While selecting enhancers, one has to account for the presence of hydroxy-groups in aromatic compounds, which upon oxidation form unstable phenoxy-radicals undergoing condensation [46]. Noteworthy, introduction of substituents such as alkyl, phenyl, amino-groups, should result in stabilization of intermediate radical products of oxidation. In addition, electron-donor substituents decrease the redox potential, while electron acceptors such as carbonyl and sulfo-groups, increase the potential. For the purposes of xenobiotic degradation, the redox potential of an enhancer has to be higher than 450 mV [35,36].

3.2. Substrate specificity

Twenty compounds of different structure (heterocyclic, derivatives of benzoic acid, and >*N*-OH compounds) have been studied; among these, only 1-phenyl-3-methyl-pyrazolone-5 and its sulfo- and amino-derivatives, *N*-hydroxyphthalimide, and 3-(6-hylroxy)-aminobenzoic acid exhibited the properties of laccase substrates (Fig. 1). Steady-state kinetics of homogeneous enzymatic reactions with the selected substrates obeys classic Michaelis–Menten equation for the dependence of initial reaction rate on the substrate concentration. To evaluate the efficiency of the selected compounds, the kinetic constants for the reaction of their oxidation with laccase have been compared with that for the best laccase substrate, hydroquinone. Table 1 presents the values of catalytic constants (k_{cat}) for the oxidation of the selected compounds, Michaelis constants (K_M), and their ratio, the characteristic of the catalytic process efficiency. Our data shows that laccase interaction with PP has a lower rate constant and a higher Michaelis constant than the reaction with HQ (Table 1). Thus, the efficiency of PP oxidation by laccase is much lower than that of HQ. We can speculate that it is the result of significant hydrophobicity of this compound which interferes with its binding to the enzyme active center. As could be seen from Table 1, phenyl-pyrazolone derivatives containing sulfo-group in para- or meta-positions of the phenyl ring, or amino-group in the heterocycle, are laccase substrates with properties comparable to those of hydroquinone.

Introduction of electron-donor groups into PP structure, e.g. 4-amino-group and 2-methyl-group (PPA), results in an 18-fold increase of k_{cat}/K_M ratio as compared to PP (Table 1).

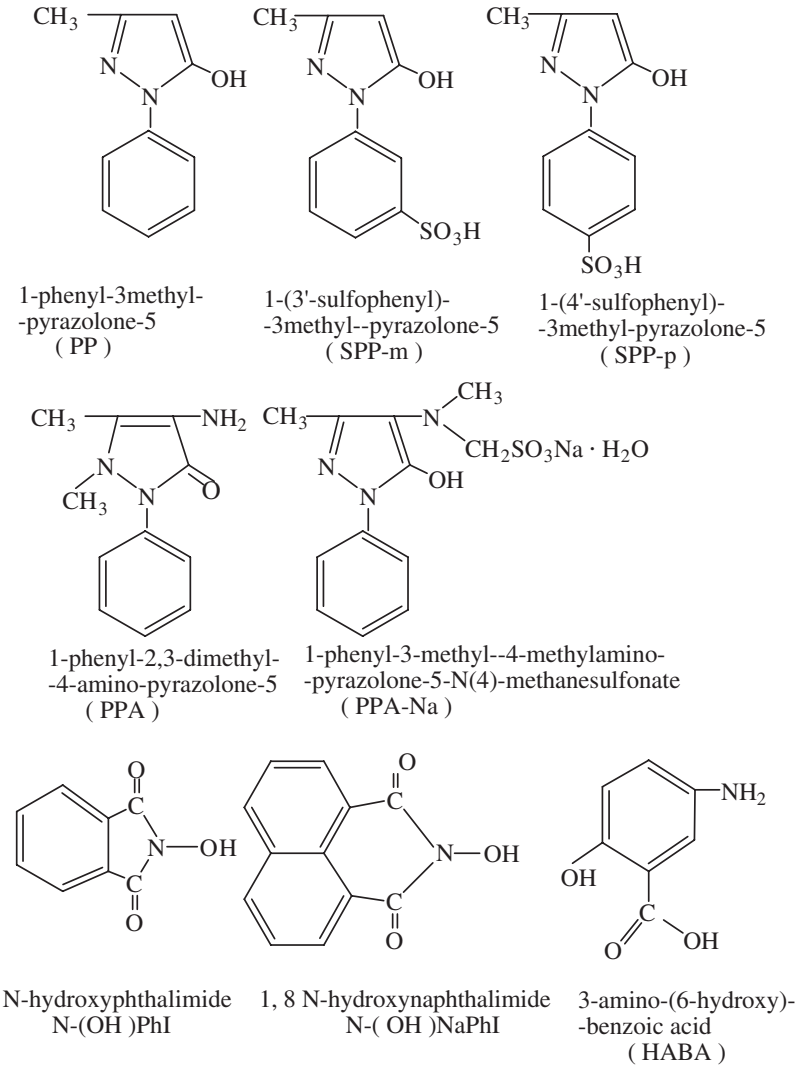

Fig. 1. Structural formulas of laccase enhancers studied.

However, this value is still 3 times lower than that for sulfo-derivative of phenyl-pyrazolone. On the other hand, introduction of substituents (methyl and methanesulfonate) into the amino-group of PPA has negligible effect on the reaction rate, but increases K_M more than 10-fold compared to *EE*A. It is likely that the weak interaction of laccase with PPA-Na is the result of spatial interference.

Kinetic parameters for the another structural series, the derivatives of benzoic acid, e.g. 3-(6-hydroxy)-aminobenzoic acid, 3-(6-hydroxy)-chlorobenzoic acid, 6-(3-chlor)-aminobenzoic acid, 4-(*N*-urea)benzoic acid, have been obtained. Only 3-(6-hydroxy)-aminobenzoic acid containing two substitutions, hydroxyl- and amino-groups, among the studied compounds is the substrate for laccase. The efficiency of homogeneous catalytic oxidation of HABA is comparable to HQ efficiency (Table 1). The replacement of hydroxyl-group for chloro-group in the structure of HABA results in a 20-fold decrease in the catalytic rate. No interaction is observed for laccase and two other compounds of this series, namely 3-(6-hydroxy)-chlorobenzoic acid and 4-(*N*-urea)benzoic acid. Table 1 also shows the catalytic constants for *N*-(OH)PhI oxidation. Laccase interaction with this compound is characterized with both lower enzyme affinity and reaction rate. Another compound of this series, *N*-hydroxynaphthalimide, is not a substrate for *T. hirsuta* laccase.

3.3. Electrochemical studies

The above-described kinetic studies of homogeneous laccase-catalyzed reactions allowed us to select prospective candidates for laccase enhancers and perform their voltammetric characterization on the glassy carbon electrode. Cyclic voltammograms for two compounds of different structure are shown in Fig. 2A and B. The potentials of the half-height of the anodic peak for the laccase substrates studied and for *N*-OH-NaphI are summarized in Table 1. Voltammograms for PP and its derivatives clearly show oxidation currents in the range of 450–630 mV (see additional data for some derivatives in [36]). As one could expect the nature of the substituent in the PP structure effects on the potential of the half-height of the anodic peak (Table 1): it decreases upon introduction of an electron-donor group into the heterocycle and increases upon introduc-

Table 1
Kinetic (k_{cat}, K_M, k_{cat}/K_M) and electrochemical ($E^{1/2}$ and $\Delta I/I_{dif}$) parameters for laccase substrates

Substrates	k_{cat} (s^{-1})	K_M (mM)	$k_{cat}\cdot 10^{-4}/K_M$ (M^{-1} s^{-1})	$E_{1/2}$ (mV)	$\Delta I/I_{dif}$
HQ	52.0	0.3	17.3	−20	–
PP[a,b]	9.7	2.1	0.46	430	0.2
SPP-m[b]	51.5	0.22	23.4	560	1.0
SPP-p[b]	43.9	0.29	15.0	570	0.8
PPA[b]	35.1	0.41	8.66	410	0.7
				550	
PPA-Na[b]	36.5	5.0	0.73	490	–
				830	
N-OH-PhI[a,c]	3.6	6.0	0.06	870	2.5
N-OH-NaPhI[c,d]	–	–	–	928	1.7
HABA	43.4	0.28	15.5	390	–

$E_{1/2}$ is a half wave redox potential for the substrate oxidation (scan rate of 5 mV/s); ΔI is an increase in the anodic current for the oxidation of an individual mediator (0.2 mM), I_{dif}, in the presence of VOH (2 mM).

[a] Oxidation of mediator by laccase was carried out in the solution containing 2% ethanol due to poor solubility of the mediator.

[b] Determination of ΔI and I_{dif} was carried out at 1000 mV.

[c] Determination of ΔI and I_{dif} was carried out in the maximum of the oxidation current for the mediator; scan rate 5 mV/s.

[d] Cyclic voltammograms were recorded in the solution containing 2% ethanol.

tion of an electron-acceptor sulfo-group into the phenyl ring. The electrode process for all PP derivatives is diffusion-limited as judged by the linear dependence of the maximum anodic current on the square root of the potential scan rate.

In addition to the anodic peak with the potential maximum at 450 mV, PPA voltammogram shows an anodic peak shoulder at 590 mV (data not shown). Upon increase in the scan rate, the ratio of the maximum currents for these peaks also increases and reaches ca. 1 at the scan rate of 100 mV/s. We speculate that at slow rates, an unstable primary product of oxidation undergoes subsequent chemical conversion, while at high scan rates it is further oxidized at the electrode. Voltammograms for sulfo-derivatives demonstrate an additional shoulder in the range of 850 mV, which is better visualized at the scan rate of 200 mV/s (Fig. 2A, arrow). It can be suggested that the shoulder is relevant to the partial oxidation of the primary product of the electrode reaction. The voltammogram for PPA-Na at the scan rate of 5 mV/s, in addition to the 570 mV peak and a 770 mV shoulder, shows a 920 mV peak comparable to that of 570 mV [36]. It is likely that the rate of the further electrochemical oxidation of the product formed at 570 mV is significantly higher than the rate of its chemical conversion. This difference in the electrochemical behavior of PPA-Na compared to other derivatives of the same series can be explained by the different SO_3-group position (not in the phenyl ring).

The reaction of oxidation of all derivatives of this series on the glassy carbon electrode is almost irreversible. This is supported by the absence of any Faraday reaction of electro-reduction on the cathodic wave of the cyclic voltammograms. Insignificant cathodic currents, 4–6-fold smaller than anodic ones, are observed on the voltammograms of PPS-m and PPA-Na. The shape of the anodic wave, which height is diffusion-limited, and the poor dependence of the potential for the anodic current maximum on the scan rate correspond to the quasi-reversible electron transfer. Irreversible oxidation of these compounds is dictated by the chemical instability of the pyrazolone oxidation product undergoing chemical transformation in the vicinity of the electrode surface.

Cyclic voltammograms for one of the compound of >*N*-OH series, *N*-hydroxyphthalimide, obtained at different scan rates, are shown in Fig. 2B. At a 5 mV/s scan rate, an anodic peak at 910 mV and a poorly visible, cathodic peak at 830 mV are observed. Upon increase in the scan rate, the reduction current increases faster than the anodic current. The ratio between the maximum cathodic and anodic peaks I_c/I_a at 100 mV/s is equal to 0.7. The position of potentials corresponding to the maximum current is almost independent of the scan rate, indicating the reaction conditions close to reversible. The difference in potentials for the maximum of anodic and cathodic peaks (ΔE) is 140 mV. Thus, at high scan rates, the system becomes quasi-reversible. The midpoint redox potential for cathodic and anodic peaks is 870 mV and it is likely to be the redox potential for this pair. At low scan rates, the unstable product, formed upon oxidation, is converted chemically faster than electrochemically. This intermediate product could be >*N*-

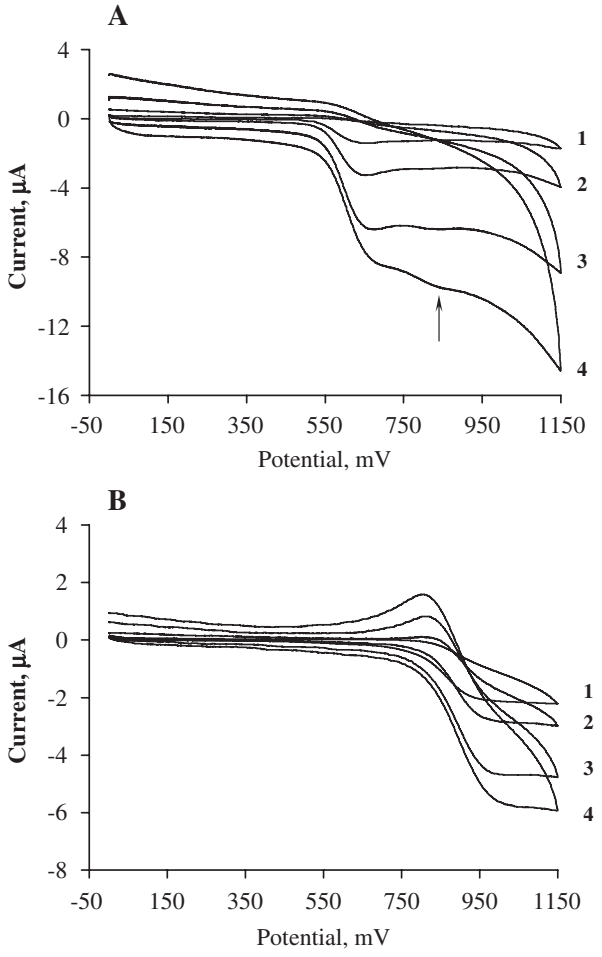

Fig. 2. Cyclic voltammograms of SPP-m (A) and *N*-OH-PhI (B) (0.2 mM) at the scan rates: (A) 1, 5 mV/s; 2, 25 mV/s; 3, 100 mV/s; 4, 200 mV/s; a shoulder in the region of 850 mV is marked by an arrow. (B) 1, 5 mV/s; 2, 25 mV/s; 3, 100 mV/s; 4, 200 mV/s. 0.1 M citrate-phosphate buffer, pH 5.0.

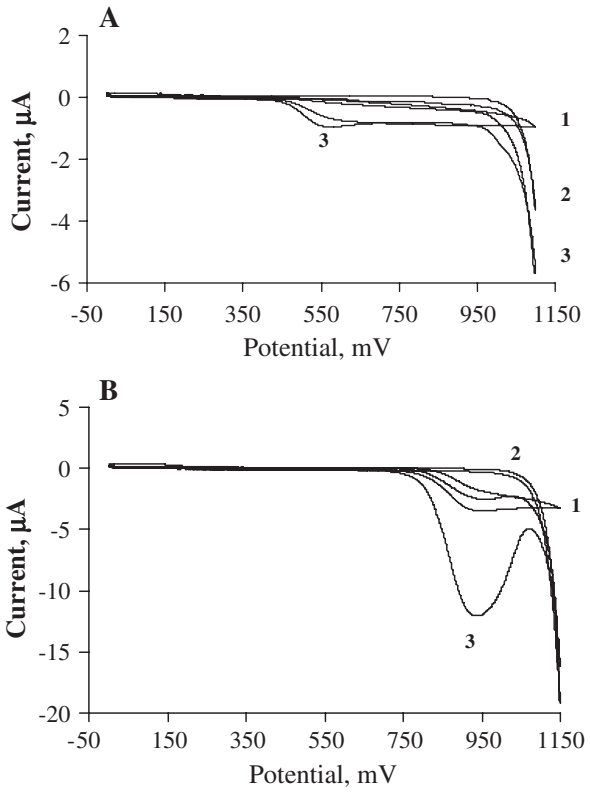

Fig. 3. Cyclic voltammograms of SPP-m (A) or *N*-OH-PhI (B) (0.2 mM), VOH (2 mM), and their mixture at a scan rate of 5 mV/s. 1, Enhancer; 2, VOH; 3, mixture. 0.1 M citrate-phosphate buffer, pH 5.0.

$O^{\bullet}$ radical like it has been shown for HBT and other >*N*-OH compounds [16,34,47]. >*N*-$O^{\bullet}$ radical is more stable than the oxidation products of pyrazolone derivatives.

The dependence of anodic and cathodic currents on the square root from the scan rate is linear. For the anodic current, the dependence can be presented by the equation:

$$I\ (\mu A) = 0.5(\pm 0.19) + 1.29(\pm 0.04)v^{1/2}\ (V/s)^{1/2},$$

where v is the scan rate.

This type of dependence proves the diffusion-limited character of the process. In accordance with the Randles-Sevcik equation [48], the number of electrons transferred can be determined as 0.8 for the compound with the diffusion coefficient (D) equal to $0.58 \cdot 10^{-5}$ cm^2/s. It is known that the values of the diffusion coefficients for the organic compounds with the MW close to that of *N*-(OH)PhI are varied in the range $0.55–0.62 \cdot 10^{-5}$ cm^2/s [49]. Using the calculated electrochemical parameters and the method proposed by Nicholson [50] for quasi-reversible processes, we calculated the heterogeneous rate constant for the electron exchange reaction of *N*-(OH)PhI on the glassy carbon electrode:

$$K_e = \Psi[D\pi v(nF/RT)]^{1/2}$$

where Ψ is a function dependent on ΔE and determined from the table [50]. At the scan rate of 100 mV/s, K_e is equal to $1.9 \cdot 10^{-3}$ cm/s. The voltammogram for the other compound of this series, *N*-(OH)NaphI, which is not a substrate for laccase, also shows an anodic peak, which is totally irreversible in contrast to that of *N*-(OH)PhI. Probably, the radical product of *N*-(OH)NaphI oxidation is less stable at the electrode than the oxidation product of *N*-(OH)PhI. The potential for the peak maximum is 90 mV higher than the corresponding potential for *N*-(OH)PhI and it is equal to 1040 mV.

Electrochemical studies of 3-(6-hydroxy)-aminobenzoic acid (HABA), which exhibited good kinetic characteristics in laccase-catalyzed reactions, demonstrated the irreversible oxidation at low scan rates (5 mV/s), i.e. only one anodic peak at 380 mV. At the scan rate of 100 mV/s, the process tends to be reversible, because the rate of electrode process becomes higher than the rate of chemical conversion of the oxidation product. The voltammogram, in addition to the anodic peak, shows the reduction peak close to 320 mV for the oxidized form of the compound, and the difference between potentials for the maximum of the anodic and cathodic peaks is 130 mV. The midpoint potential is equal to 390 mV.

3.4. Electrochemical oxidation of veratryl alcohol in the presence of enhancers

Cyclic voltammetry was applied to study the reaction of electro-oxidation of lignin model compound, veratryl alcohol (VOH), in the presence of the novel laccase substrates studied above. This method allows us to investigate not only the electrode processes but also the chemical reactions initiated by them. Fig. 3A shows cyclic voltammograms recorded in 2 mM solution of veratryl alcohol, in 0.2 mM solution of 1-(3′-sulfophenyl)-3methyl-pyrazolone-5, and in their mixture in the same concentrations. VOH is electrochemically inactive up to potentials of ca. 1000 mV. In the presence of sulfo- and amino-derivatives of pyrazolones, the anodic current shows the oxidation wave in the potential range of 1000 mV, in addition to the oxidation current of the mediator itself in the range of 430–570 mV (depending on the nature of the PP derivative). An increase in the concentration of the mediator results in the

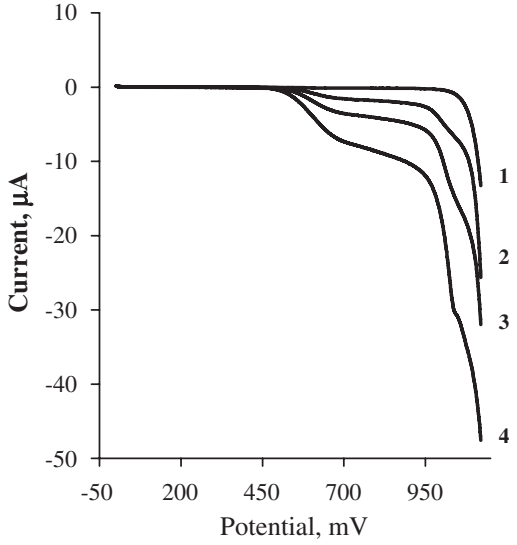

Fig. 4. Anodic voltammograms (scan rate of 5 mV/s) recorded in the solutions of VOH (4 mM) and SPP-p at the concentration: 1, 0 mM; 2, 0.5 mM; 3, 1.3 mM; 4, 2.8 mM. 0.1 M citrate-phosphate buffer, pH 5.0.

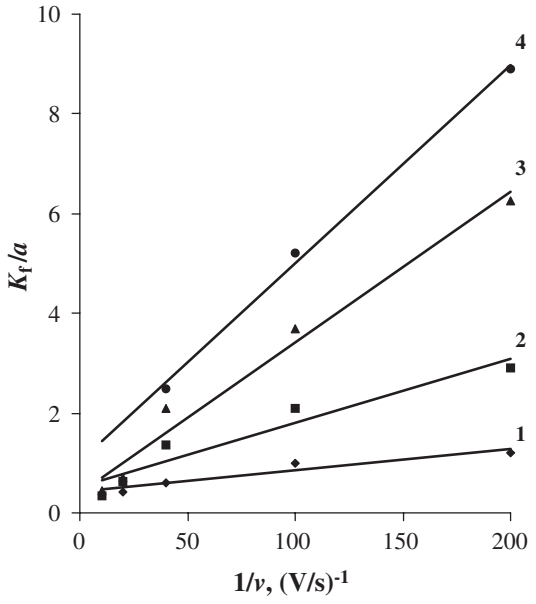

Fig. 5. Dependence of the kinetic parameter (k_f/a) for VOH oxidation in the presence of *N*-(OH)PhI on a GC electrode vs. $1/v$ at VOH concentrations: 1, 0.5 mM; 2, 1 mM; 3, 2 mM; 4, 3 mM; where a is nFv/RT, and v is a scan speed. 0.2 mM *N*-(OH)PhI, 2 mM VOH in 0.1 M citrate-phosphate buffer, pH 5.0.

increase of this oxidation wave (Fig. 4). These results demonstrate oxidation of veratryl alcohol in the presence of oxidation product of PP derivative (except PPA-Na) with lower overvoltage than the direct electrode reaction. It should be emphasized that the potential of irreversible electrochemical oxidation of veratryl alcohol on the glassy carbon electrodes in the absence of an enhancer is close to 1230 mV. An increase in anodic current at the potential of 1000 mV in the solution containing VOH and the enhancer compared to the diffusion current of the enhancer oxidation at the same potential (I_{dif}) in the solution without VOH will be further referred to as ΔI. The ratios $\Delta I/I_{dif}$ for the studied pyrazolone derivatives are presented in Table 1. As can be seen, the compounds of pyrazolone group with the half-oxidation potential higher than 430 mV are effective in the reaction with VOH. The effectiveness of electrocatalytic oxidation of VOH increases with the increase in the potential of oxidation half-wave of the enhancer.

Individual voltammograms for VOH and *N*-(OH)PhI and for their mixture are presented in Fig. 3B. For all scan rates used in this work, a significant increase in anodic current in the range of *N*-(OH)PhI oxidation potentials is observed in the presence of VOH linked to the complete disappearance of reduction current in the cathodic wave of the curve. This disappearance can be explained by the involvement of the intermediate of *N*-(OH)PhI electrochemical oxidation into the process of chemical oxidation of VOH in the close vicinity of the electrode. The chemically reduced intermediate is repeatedly oxidized by the electrode resulting in the increased anodic current compared to that of *N*-(OH)PhI in the absence of VOH. At the scan rate of 5 mV/s, the anodic current at the potential of 910 mV in the presence of VOH increases 2.5-fold compared to the current in the absence of VOH (Fig. 3B). The experimental results showing the interrelationship between the current and scan rate support the diagnostic criterion proposed by Nicholson and Shain [51] and used to reveal the coupling of electrode and catalytic processes. In particular, the $I_{max}/v^{1/2}$ ratio increases with a decrease in v, approaching some constant value (v is the scan rate). Thus, the process at the electrode can be described by the following scheme:

$$R - e^- \leftrightarrow O \quad [VOH]_{red} + O \xrightarrow{kf} R + [VOH]_{ox,}$$

where R and O are reduced and oxidized forms of the enhancer, respectively, $k_f = k_{ch}[VOH]$ (k_{ch} is the rate constant for the first order chemical reaction).

Noteworthy, the heterogeneous electron transfer in this system occurs faster than the coupled homogeneous electron transfer. The method proposed in [52] allows us to calculate the rate constant for the chemical reaction. In accordance with the theoretical model for the reactions proceeding via such scheme, the experimental parameter $\Delta I/I_{dif}$ is equal to the kinetic parameter $(k_f/a)^{1/2}$, where a is nFv/RT, v is the scan rate and n, F, R, and T have their usual meaning. Fig. 5 shows the dependence of k_f/a on $1/v$ for varied concentrations of veratryl alcohol. The data in Fig. 5 can be presented as a linear dependence of parameter $k_f RT/nF$ on the concentration of VOH. This dependence gives the rate constant of pseudo-first order, $k_{ch} = k_f/[VOH]$, which is equal to 280 M^{-1} s^{-1}. This value is comparable to the constant of the veratryl alcohol oxidation on the electrode in the presence of ABTS [34]. It can be suggested that the high potential of the intermediate formed upon electrode oxidation of *N*-(OH)PhI provides higher rates of veratryl alcohol oxidation. Although the electrochemical oxidation of another high potential compound (*N*-hydroxynaphthalimide) is irreversible, the electrocatalytic oxidation of VOH in its presence does take place, but the $\Delta I/I_{dif}$ ratio is smaller than that for *N*-(OH)PhI. On the contrary, the stimulation of VOH electro-oxidation is not observed in the presence of the low-potential product (redox potential <450 mV) of HABA electrode oxidation.

3.5. Veratryl alcohol oxidation in the laccase-redox enhancer system

We concluded that the electrocatalytic oxidation of veratryl alcohol requires the enhancer oxidation intermediates with the potentials of half-oxidation higher than 500 mV. To clarify the effectiveness of the selected enhancers in the degradation of lignin model compound, veratryl alcohol, we performed direct

Table 2
Oxidation of veratryl alcohol mediated by different laccase-mediator systems

Substrates	VOH conversion degree, 48 h incubation (%)
SPP-m	35
SPP-p	30
PPA-Na	4
N-OH-PhI	73
ABTS	76

Initial veratryl alcohol concentration of $1 \cdot 10^{-3}$ M; $5 \cdot 10^{-3}$ M mediator; $1 \cdot 10^{-6}$ M *Trametes hirsuta* laccase. The S.E.M. calculated from three independent experiments did not exceed 15%.

experiments: the system containing 1 mM VOH, 5 mM redox-enhancer, and 1 μM laccase was incubated for 48 h at 20 °C followed by HPLC detection of the reaction products.

The results are summarized in Table 2. The highest conversion degree comparable to that of ABTS-mediated oxidation was obtained for *N*-OH-PhI-mediated laccase-catalyzed oxidation of VOH to veratryl aldehyde (VCOH). At the same time, in the catalytic system laccase–SPP-m and SPP-p the conversion degree of VOH to VCOH was 35% and 30%, respectively. The lower conversion degree of VOH in this case is probably the result of poor reversibility of oxidation process for pyrazolone-5 derivatives, which was observed in our electrochemical studies. As one may expect from voltammetric studies, the rate of VOH oxidation in the laccase–PPA-Na system appeared to be much lower than with SPP-m and SPP-p. The main biodegradation product of VOH is VCOH. In addition, using PPA-Na as an enhancer, we detected minor amounts of veratric acid (3,4-dimethoxybenzoic acid) as one of the VOH oxidation products [36].

The electrochemical studies show that the redox potential of the >*N*-OH/>*N*-O$^{\bullet}$ system is higher than the redox potential of *T. hirsuta* laccase by 290 mV. However, since this system is quasi-reversible under some specified conditions, slow oxidation of *N*-OH-PhI by laccase is thermodynamically permitted. This has been shown by kinetic measurements: high potential >*N*-O$^{\bullet}$ radical generated electrochemically is able to catalyze VOH oxidation with rather high rates. Cation radical formed in the presence of laccase is also capable of oxidizing VOH. Due to the high rate of VOH oxidation by the cation radical, the overall effectiveness of VOH degradation by laccase–*N*-OH-PhI system is high and results in a 70% conversion degree. A different issue is the laccase systems with 1-phenyl-3-methyl-pyrazolone-5 derivatives. Kinetic studies show that the enzyme catalyzes the oxidation of amino- and sulfo-derivatives with high rates. The electrochemical studies demonstrated the oxidation of these derivatives with high rates, but the oxidation products are unstable. However, the potential and lifetime of the oxidation product are sufficient to catalyze slow oxidation of VOH. Due to the high rates of enzymatic oxidation of these derivatives, the overall effectiveness of VOH oxidation in the catalytic system laccase-enhancer is high enough and reaches a 35% conversion degree for VOH.

We conclude that 1-phenyl-3-methyl-pyrazolone-5 derivatives can be successfully used as laccase enhancers for the enzymatic degradation of xenobiotics including lignin-type compounds. Accounting for the non-recoverable loss of organic mediators during the laccase-catalyzed enzymatic reaction, production costs are of primary importance. Production cost for 1-phenyl-3methyl-pyrazolone-5 derivatives is 2–3 orders of magnitude lower than that of ABTS. This makes the newly discovered laccase enhancers extremely prospective for commercial applications.

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