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Novel Laccase Redox Mediators

Spectral, Electrochemical, and Kinetic Properties

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Abstract

The screening of potential redox mediators for laccase was performed using homogeneous enzyme preparations from *Coriolus hirsutus* and *Coriolus zonatus*. It was discovered that derivatives of 1-phenyl-3-methyl-pyrazolones were efficient substrates for the laccases. The characterization of two representatives of the 1-phenyl-pyrazolone class, sodium 1-phenyl-3-methyl-4-methylamino-pyrazolone-5-*N*(4)-methanesulfonate and 1-(3'-sulfophenyl)-3-methylpyrazolone-5, in the reaction catalyzed by laccase was carried out using spectral, electrochemical, and enzyme kinetics methods. The kinetic parameters for the oxidation of the newly discovered substrates were comparable with those for 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) oxidation by laccase. Electrochemical experiments demonstrated that oxidation of these compounds yielded two high-potential intermediates capable of oxidizing veratryl alcohol, which was used as a lignin model substrate, to the corresponding aldehyde and acid. 1-(3'-Sulfophenyl)-3-methylpyrazolone-5 was about 30–40% as effective in degrading veratryl alcohol compared to ABTS as judged from high-performance liquid chromatography kinetic studies. 1-Phenyl-3-methyl-pyrazolones may be of commercial interest for oxidoreductase-catalyzed biodegradation of organic compounds.

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Index Entries: Laccase; mediator; lignin; redox potential; 1-phenyl-3-methyl-pyrazolone.

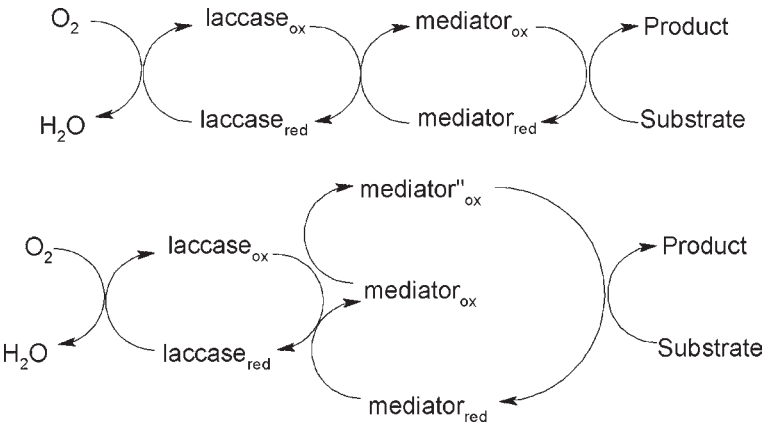
Introduction

A number of oxidoreductases, such as peroxidases, tyrosinases, ligninnases, and laccases, are of high potential for commercial biotechnology processes. Recent studies on lignin biodegradation *(1)* have shown that laccase is the most important component of the natural lignin-degrading fungal system. Laccase (EC 1.10.3.2) catalyzes the oxidation of a wide spectrum of organic compounds such as *o*- and *p*-diphenols, aminophenols, polyphenols, polyamines, heterocyclic compounds, and some inorganic ions coupled with the reduction of molecular dioxygen to water *(2)*. The catalytic properties of laccase make it a promising biocatalyst for applications in biofuel cells *(3)*, analytical systems *(4,5)*, organic waste detoxification *(6,7)*, and organic synthesis *(8,9)*.

Numerous plant and fungal laccases have been characterized *(2,10)*. They have four Cu ions of three types, which are classified as the so-called blue copper (T1), type 2 copper (T2), and a binuclear center (T3) *(2,11)*. The oxidation of organic compounds catalyzed by laccase occurs via a common ping-pong mechanism. However, the reaction products are highly reactive and may launch free-radical chain reactions *(2,11,12)*. Therefore, the enzymatic process is usually accompanied by nonenzymatic radical polymerization or polycondensation. Thus, laccase participates in both lignin biodegradation and protection of the fungal mycelium by polymerizing toxic products of lignin biodegradation. The recently reported production of 29-kDa polymers catalyzed by laccase *(8,9)* illustrates the synthetic potential of this enzyme.

The wide substrate specificity of laccase can be enhanced by using so-called redox mediators. The use of redox mediators that have only one oxidation state allows laccase to overcome a kinetic barrier, while the use of redox mediators that can undergo subsequent laccase-catalyzed oxidations and/or disproportionate yielding even more active radical species, such as 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) dication, allows compounds with redox potentials higher than that of the enzyme to be oxidized (cf. Scheme 1 top and bottom).

The key requirement for a mediator is the reversibility of its oxidation/reduction, which is mainly based on the stability of the radical in air. The effectiveness of the discussed systems has been shown for numerous practical applications in different biotechnology fields *(6,7,10,13,14)*. Current studies are focused on screening and selection of laccase producers, optimization of cultivation, fundamental research on the nature of enzyme-active centers and catalytic mechanism, and screening for new substrates and mediators. Major requirements for mediator properties in the particular case of lignin biodegradation are a high number of redox cycles, no secondary polymerization, and the nontoxicity of colorless oxidation products. The most popular among currently studied mediators



Scheme 1. Laccase-catalyzed oxidation with meditor cycling between two (top) and three (bottom) redox states.

are ABTS, 1-hydroxybenzotriazole (HBT), 2,2,6,6-tetramethyl-l-piperidinyl-oxyl *(6,10,15)*, and some organic and inorganic complexes of transition metals *(16)*. However, there is still a need to develop novel mediators because the existing ones either are "false" mediators, i.e., are consumed or degraded in the reaction course, or are too expensive for commercial applications.

The goal of the present study was to develop a rational approach for the selection of laccase redox mediators and their complete characterization in laccase-catalyzed reactions.

Materials and Methods

Chemicals

2-Mercaptoethanol was from Ferak (Berlin, Germany); Tris and glycine were from ICN (Aurora, OH); acrylamide, *N*,*N'*-methylenebisacrylamide, and ammonium persulfate from Reanal (Budapest, Hungary) were purified by crystallization; ABTS and catechol were from Sigma (St. Louis, MO); 3,4-dimethoxybenzyl alcohol, 3,4-dimethoxybenzaldehyde, and 3,4-dimethoxybenzoic acid were from Aldrich (Milwaukee, WI); and 2,2'-biquinoline and Na_2HPO_4 were from Merck (Darmstadt, Germany). Glycerol, acetic acid, methanol, ethanol, sodium 1-phenyl-3-methyl-4-methylamino-pyrazolone-5-*N*(4)-methanesulfonate (PPNa), 3-sulfophenylhydrazine, H_3PO_4, HCl, NaOH, KH_2PO_4, NaCl, KCl, and $(NH_4)_2SO_4$ were of the highest purity available from domestic sources. 1-(3'-Sulfophenyl)-3-methylpyrazolone-5 (SPP) was synthesized by condensation of 3-sulfophenylhydrazine and acetyl acetate at 55–60°C in water solution in accordance with ref. *17*.

Enzymes

Strains *Coriolus hirsutus* and *Coriolus zonatus* producing extracellular laccase were obtained from the laboratory collection of the Federal State

Unitary Enterprise "GosNIISintezbelok." Cultivation was performed in accordance with refs. *18* and *19*. The purification scheme included enzyme precipitation from the cultural medium with saturated ammonium sulfate, fine performance liquid chromatography (FPLC) ion-exchange chromatography on Servacel DEAE 52 (Reanal) and rechromatography on DEAE-Toyopearl 650M (Toyo Soda, Tokyo, Japan) performed on standard equipment purchased from LKB (Uppsala, Sweden), and isoelectric focusing in the pH range of 3.0–5.0, followed by gel filtration on Toyopearl HW-55 (Toyo Soda). Preparative isoelectrofocusing was performed using a pH gradient of 3.0–5.0 stabilized by a glycerol gradient (10–60%) on a 440-mL column 8100 (LKB). The power applied was 30 W at the beginning of the experiment and 3 W at the end. Fractions were collected at a flow rate of 4 mL/min. The final purification was performed by means of high-performance liquid chromatography (HPLC) on a TSK DEAE-2SW column (LKB) using a Stayer-system (Aquilon, Moscow, Russia). The enzyme preparation exhibited a specific activity of 350 U/mg with catechol as a substrate.

The enzyme preparations were homogeneous as judged from sodium dodecyl sulfate polyacrylamide gel electrophoresis. The molecular mass of laccase was determined by gel filtration on a TSK G 2000 SWXL (TosoHaas, Stuttgart, Germany), calibrated with a standard set of 15- to 92-kDa markers from Serva (Heidelberg, Germany). Laccase-bound copper was estimated with 2,2'-biquinoline *(20)*.

Activity Assay

Laccase activity in the course of purification, pH optimum, and thermal stability measurements were performed using 10 m*M* catechol (ε = 740 $M^{-1}\cdot cm^{-1}$) as a chromogenic substrate in 0.1 *M* Na acetate buffer, pH 4.9, at 410 nm *(20,22)* on a Hitachi-557 spectrophotometer (Tokyo, Japan). The activity was expressed as micromoles of catechol oxidized in 1 min by 1 mg of protein.

Steady-State Kinetics

Determination of rate constants was performed from the data on oxygen consumption using a Clark-type electrode and a voltammetric analyzer CV-50W BAS (West Lafayette, IN) in accordance with the method described in ref. *21*. A substrate solution in 0.1 *M* Na citrate–phosphate buffer, pH 4.9 (0.5 mL), was placed in an electrochemical cell, and the reaction was initiated by the addition of laccase. The concentration of a substrate was varied within the 10^{-6}–10^{-1} *M* range, and the enzyme concentration was adjusted such that the rate of oxygen consumption was constant for the first 60 s independent of the substrate concentration used. The initial oxygen concentration was taken as equal to 260 µ*M*. All measurements were performed in triplicate at 20°C. Kinetic parameters were calculated from the Michaelis-Menten equation using an ENZFITTER version 1.05 program.

Cyclic Voltammetry

Cyclic voltammograms were recorded in 0.1 *M* Na citrate–phosphate buffer at different pH values with a CV-50W voltammetric analyzer (BAS). The scan rate varied from 10 to 200 mV/s. A glassy carbon electrode was used as a working electrode, an Ag/AgCl electrode was used as a reference, and 1-mm-diameter Pt wire was used as an auxiliary electrode. All values are expressed as potentials against an Ag/AgCl electrode.

HPLC Studies

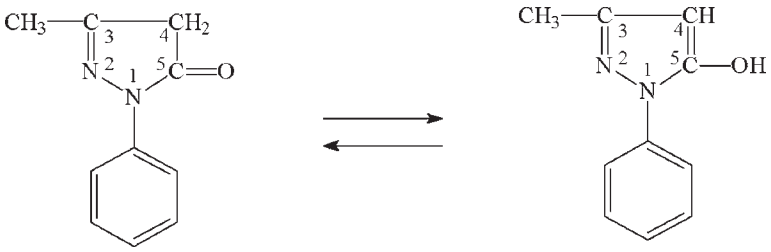
Analysis of veratryl alcohol oxidation products was performed by reverse-phase hydrophobic HPLC on a Luna C18 column (250 × 4.6 mm) (Phenomenex, Torrance, CA) with a linear gradient of acetonitrile (5–95%) in 0.086% H_3PO_4 at a 1 mL/min flow rate using a Stayer-system (Aquilon, Moscow, Russia). The incubation mixture contained 10^{-3} *M* veratryl alcohol, 5×10^{-3} *M* PPNa or SPP, and 10^{-6} *M C. hirsutus* laccase in 0.1 *M* Na phosphate–citrate buffer, pH 4.9, at 20°C. The control mixture had either laccase or mediator omitted. The sample preparation included ultrafiltration through a PM-10 membrane in an Amicon cell (Millipore, Bedford, MA) 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 (Russia).

Results and Discussion

As noted in the Introduction, the key requirement for an efficient redox mediator is the stability and high redox potential of its radical product. The radical product should readily react with the target substrate but, at the same time, should not be oxidized by air oxygen or polymerized. Taking this into account, the following screening strategy for the potential redox mediators was developed: (1) initial selection based on a chemical formula, (2) demonstration of enzymatic activity of the individual enzyme with respect to the selected compound, (3) determination of the redox potential of the oxidation intermediates of the selected compound, (4) demonstration of the effect of a target substrate on the cyclic voltammogram of the selected compound, and (5) direct HPLC analysis of target substrate decomposition in the presence of the enzyme and a selected mediator under the conditions of nonenzymatic interaction between the oxidized mediator and target substrate. Only the last step can be considered unequivocal proof of the mediator nature of the selected compound. This approach for mediator screening was used for the system with laccase as an enzyme and veratryl alcohol as a target substrate.

Chemical Structure

The mechanism of substrate oxidation by laccase includes one-electron oxidation of the substrate molecule with the oxidized enzyme form.

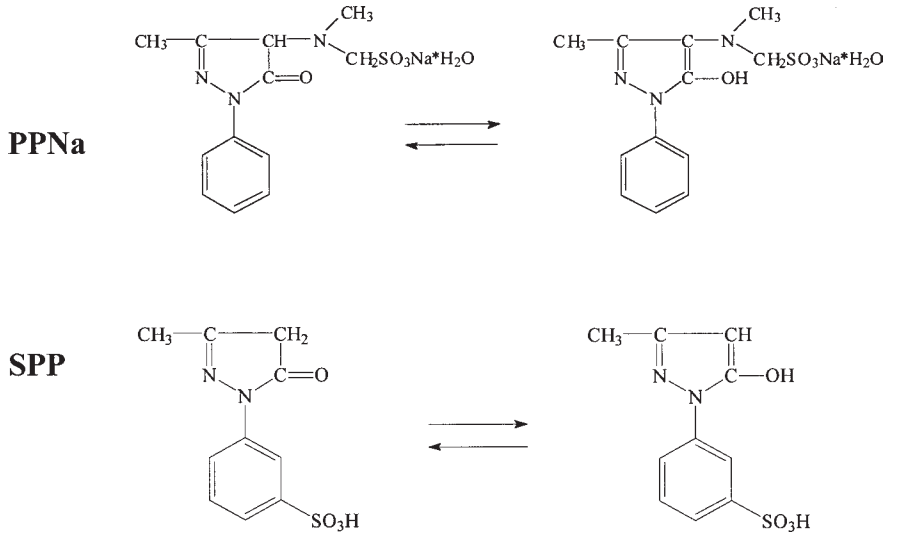


Scheme 2. Chemical structure of 1-phenyl-3-methyl-pyrazolone.

Thus, the substrate must have electron donor properties and easily form a radical product. Both aromatic and heterocyclic compounds satisfy these criteria. In the case of aromatic compounds, the benzyl ring itself is extremely stable with respect to oxidation; however, its properties are, to a large extent, determined by the nature of substituents. The presence of hydroxy groups usually leads to phenoxyradicals, which are easily oxidized by molecular oxygen with the subsequent formation of peroxo forms *(22)*. This may lead to the opening of the ring and decomposition of the substrate *(22)*. However, the formation of products by oxidative polymerization is also possible *(23)*. The presence of amino groups also results in dimerization/polymerization of the intermediate radicals such as occurs during the peroxidase-catalyzed oxidation of benzidine derivatives *(24)*. Heterocyclic rings on oxidation are also likely to incorporate oxygen and decompose. However, they may form a stable radical if coupled together in a manner analogous to the formation of pi-cation radicals of porphyrins *(25,26)* or coupled with aromatic rings such as ABTS *(27)*. Taking into account the fact that ABTS is the best organic mediator known, we can formulate the following requirements for a potential mediator: (1) heterocyclic ring(s), (2) coupled bonds or easily oxidized substitutions (e.g., hydroxyl groups), (3) electron donor substituents (e.g., phenyl, alkyl) that stabilize the generated radical, and (4) sulfo groups providing solubility in water.

To be considered of commercial importance, a potential mediator or its precursors must be already commercially available as low-cost commodity chemicals. The search for nontoxic heterocyclic products on the Russian market led us to well-known painkiller medications and dyes produced on a commercial level whose chemical structures are based on 1-phenyl-3-methyl-pyrazolone (Scheme 2).

The pyrazolone shown in Scheme 1 is a heterocycle that contains two electron-donor substituents (i.e., methyl and phenyl groups), which should stabilize the radical intermediate formed on oxidation. The key substituent providing easy oxidation is a carbonyl group, which exists in equilibrium with a tautomeric enol form, 1-phenyl-3-methyl-5-hydroxypyrazole. We selected two representatives of this class of compounds containing a sulfo group to provide water solubility: PPNa and SPP (*see* Scheme 3).



Scheme 3. Chemical structure of PPNa and SPP.

Table 1
Some Physicochemical Properties of Laccase From Basidiomycetes

Laccase source	Mol wt (kDa)	p*I*	pH optimum	Copper content	Thermal stability (residual activity [%] of laccase after 96 h of incubation at 40°C)
C. hirsutus	55 ± 3	4.05 ± 0.05	4.4 ± 0.2	4.0	41.0
C. zonatus	60 ± 3	4.65 ± 0.05	4.8 ± 0.2	3.8	47.0

Laccase-Catalyzed Oxidation of Phenylpyrazolones: Kinetics and Intermediate Formation

The properties of the homogeneous laccase preparations used to study the activity toward the newly selected compounds are summarized in Table 1. Both enzymes exhibit sufficient thermal stability and convenient pH optimum and, thus, are of interest for biotechnology purposes. Both compounds selected are good substrates for the laccase preparation used in the present study, and the rate constants for their oxidation are comparable with that of ABTS (Table 2).

The nature of the intermediates produced in the course of laccase-catalyzed oxidation of PPNa depends on the concentration ratio between laccase and PPNa in solution. Analysis of the spectral changes in the course of the oxidation reveals a number of oxidation intermediates. At a high substrate-to-enzyme ratio (10,000) two absorbance peaks at 330 and 400 nm

Table 2
Kinetic Parameters of Enzymatic Oxidation
of PPNa and SPP Catalyzed by *Coriolus* Laccase
(0.1 *M* citrate-phosphate buffer, pH 5.0)

Parameter	Substrate	*C. hirsutus*	*C. zonatus*
k_{cat} (s^{-1})	ABTS	40 ± 3	50 ± 5
	PPNa	40 ± 3	42 ± 4
	SPP	56 ± 2	45 ± 4
K_m (m*M*)	ABTS	0.05 ± 0.008	0.02 ± 0.006
	PPNa	5.0 ± 0.80	0.8 ± 0.20
	SPP	0.2 ± 0.07	0.1 ± 0.06

(Fig. 1A) are clearly visible. Comparison of the rates of increase in absorbance at 330 and 400 nm during the course of PPNa oxidation under the conditions presented in Fig. 1A shows that both peaks grow at a similar rate. Thus, these peaks are likely to correspond to one and the same oxidation intermediate. At a lower ratio (6000), formation of an intermediate product with an absorbance peak at 520 nm is observed (Fig. 1B). At a comparatively low ratio (500), the peak at 625 nm exhibits continuous growth (Fig. 1C). Thus, analysis of spectral changes in the course of the PPNa oxidation reveals at least three intermediates. If one takes into account the fact that high enzyme-to-substrate ratios lead to the production of compounds with a high redox potential, the following sequence of redox potentials for the spectral intermediates of the enzymatic oxidation of PPNa may be proposed: $P_{330} = P_{420} < P_{500} < P_{625}$ (in which *P* is the oxidation product, and the subscript is the wavelength of the absorbance peak detected).

During SPP enzymatic oxidation (Fig. 2), a decrease in the peak at 240 nm occurs, making the contribution of a peak at 270 nm more visible. This peak remains unchanged in the oxidation course while a product with an absorbance maximum at 340 nm appears to be the end product. Analysis of the time course of absorbance changes in the course of SPP oxidation leads to the conclusion that the mechanisms of SPP oxidation resemble those of ABTS oxidation, i.e., sequential oxidation *(15)*. However, the second step of the enzymatic SPP oxidation does not yield a bication like the ABTS bication, but more likely leads to a radical similar to that formed in the case of HBT enzymatic oxidation *(15)*.

Electrochemical Characterization of Oxidation Intermediates of Phenylpyrazolones

Cyclic voltammetry has been shown to be an efficient method for primary mediator screening *(15)*. The analysis is based on the determination of the redox potentials of oxidation intermediates produced in the course of electrochemical oxidation. Bourbonnais et al. *(15)* described intermediates produced by electrochemical oxidation of ABTS and HBT and determined their redox potentials. The method was demonstrated to be

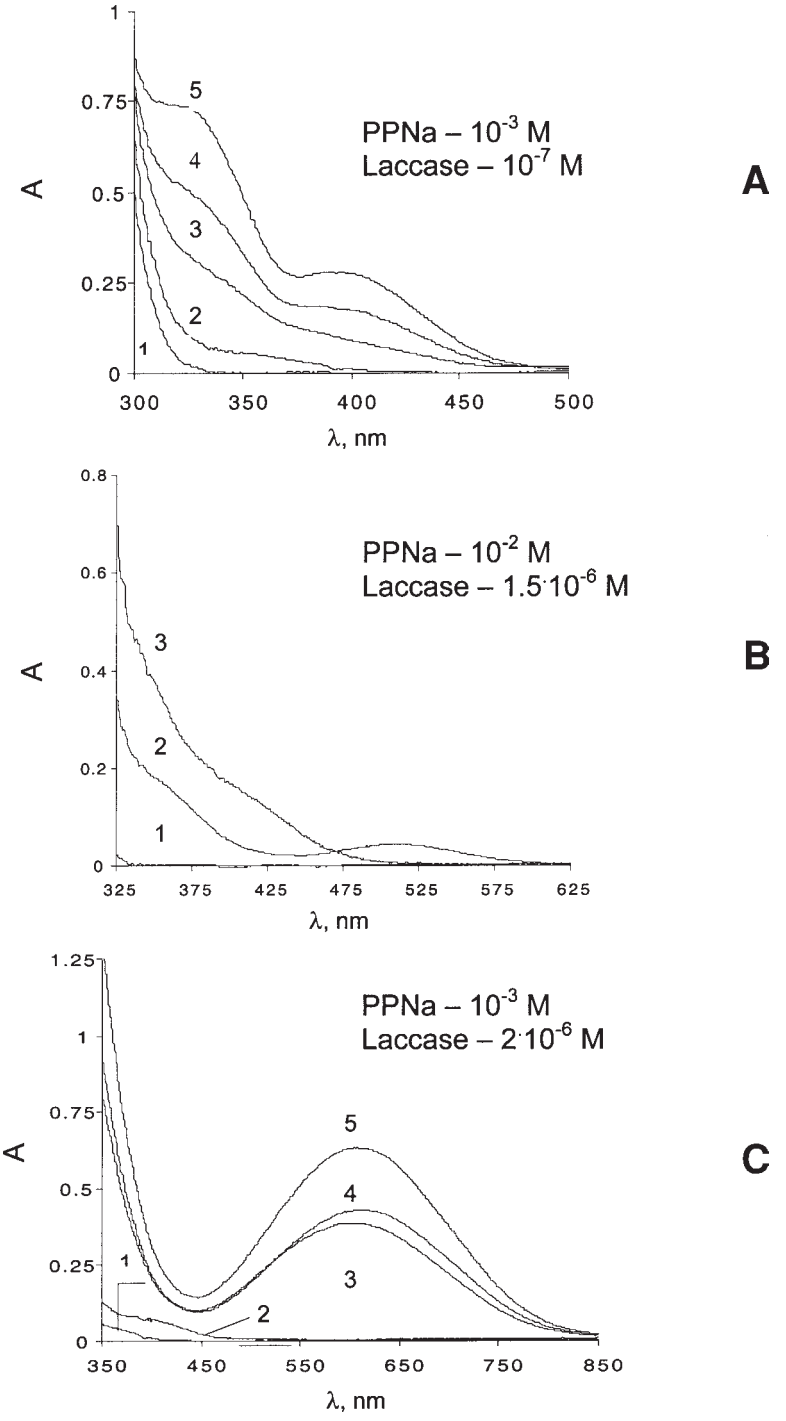


Fig. 1. Ultraviolet (UV)/visible spectra of reaction products formed during course of PPNa oxidation catalyzed by *C. hirsutus* laccase at various concentrations of mediator and enzyme. 1, initial spectrum; 2, spectrum recorded 1 min after the reaction was initiated; 3, 10 min later; 4, 30 min later; and 5, 60 min later.

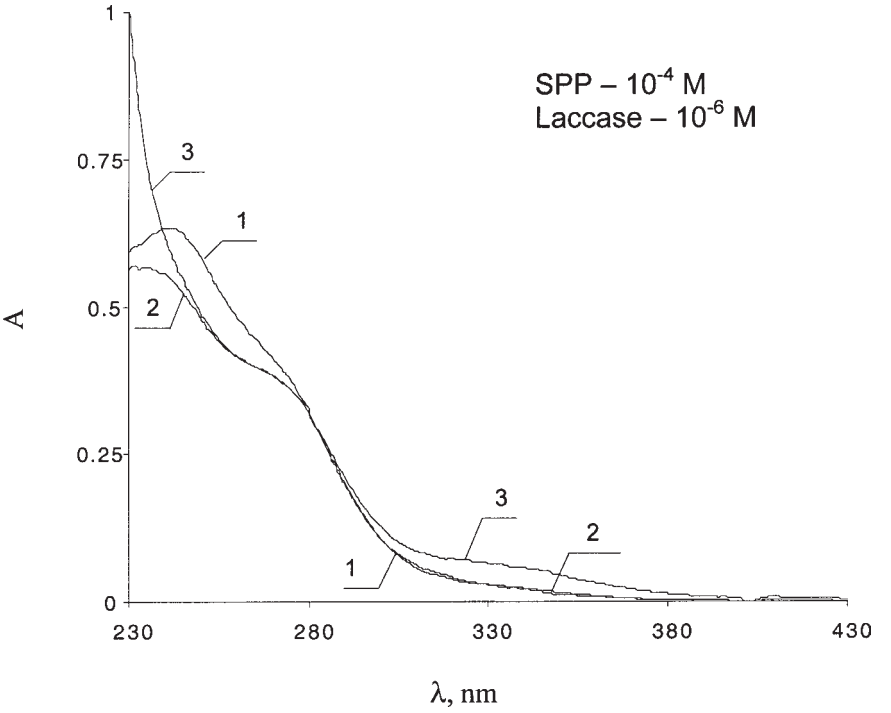


Fig. 2. UV/visible spectra of reaction products formed during course of 10^{-4} *M* SPP oxidation catalyzed by *C. hirsutus* laccase (10^{-6} *M*). 1, initial spectrum; 2, spectrum recorded 3 h later; 3, spectrum recorded 24 h later.

applicable for the primary screening of mediators. The ABTS and HBT oxidation intermediates are of sufficiently high redox potential to oxidize lignin model compounds, and veratryl alcohol in particular, resulting in a significant increase in current on polarization curves.

Cyclic voltammetry data for the selected pyrazolone compounds are presented in Fig. 3. Both compounds yield oxidation intermediates in the potential range of 0–1100 mV. The estimated redox potentials for the electrochemical oxidation products for PPNa are 290, 570, 770, 920, and 1000 mV (Fig. 3A), and 630 and 790 mV for SPP (Fig. 3B). In general, the electrochemical data agree with the spectral data obtained. It is likely that the final oxidation product observed for SPP at 340 nm has a high redox potential of 790 mV, while the first oxidation product has a redox potential of 630 mV. In the case of PPNa, the formation of the fifth oxidation intermediate with a redox potential of 1000 mV in the course of enzymatic oxidation is doubtful because the standard redox potential of blue laccase is usually no more than 800 mV *(2,6,28)*.

The oxidation of both compounds is only partial, and, moreover, some intermediates are produced irreversibly. For example, PPNa exhibits a pronounced cathodic peak with a potential of 470 mV, and a shoulder at 700 mV. In the case of SPP, the cathodic part of the polarization curve has a peak at 130 mV and a shoulder at 510 mV, which probably correspond to

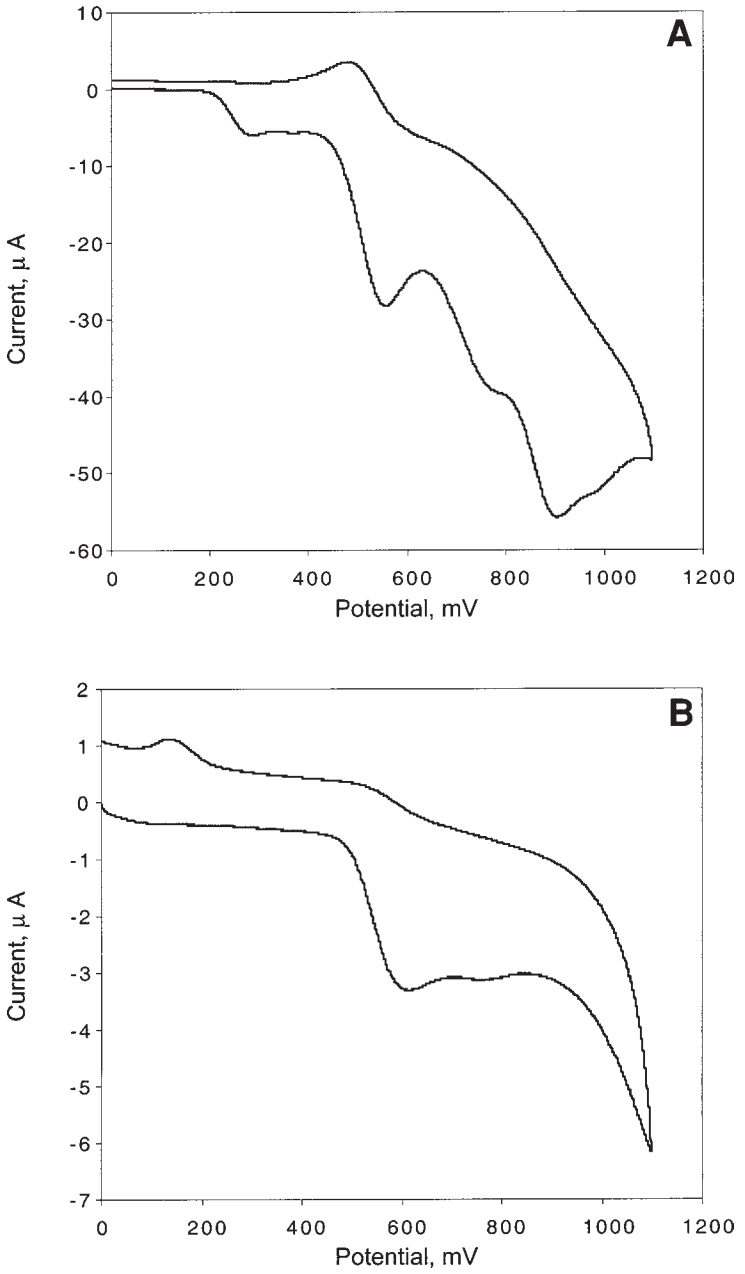


Fig. 3. Cyclic voltammograms of **(A)** PPNa and **(B)** SPP (0.1 *M* sodium citrate–phosphate buffer, pH 4.9; 0.2 m*M* mediator; scan rate of 100 mV/s). The start potential was 0 mV.

the reduction of the high redox potential intermediates formed at the anodic part of the curve. The addition of veratryl alcohol to the electrochemical cell did not result in an increase in oxidation currents at potentials lower than 1000 mV for PPNa (Fig. 4A), while for SPP the current increased at least

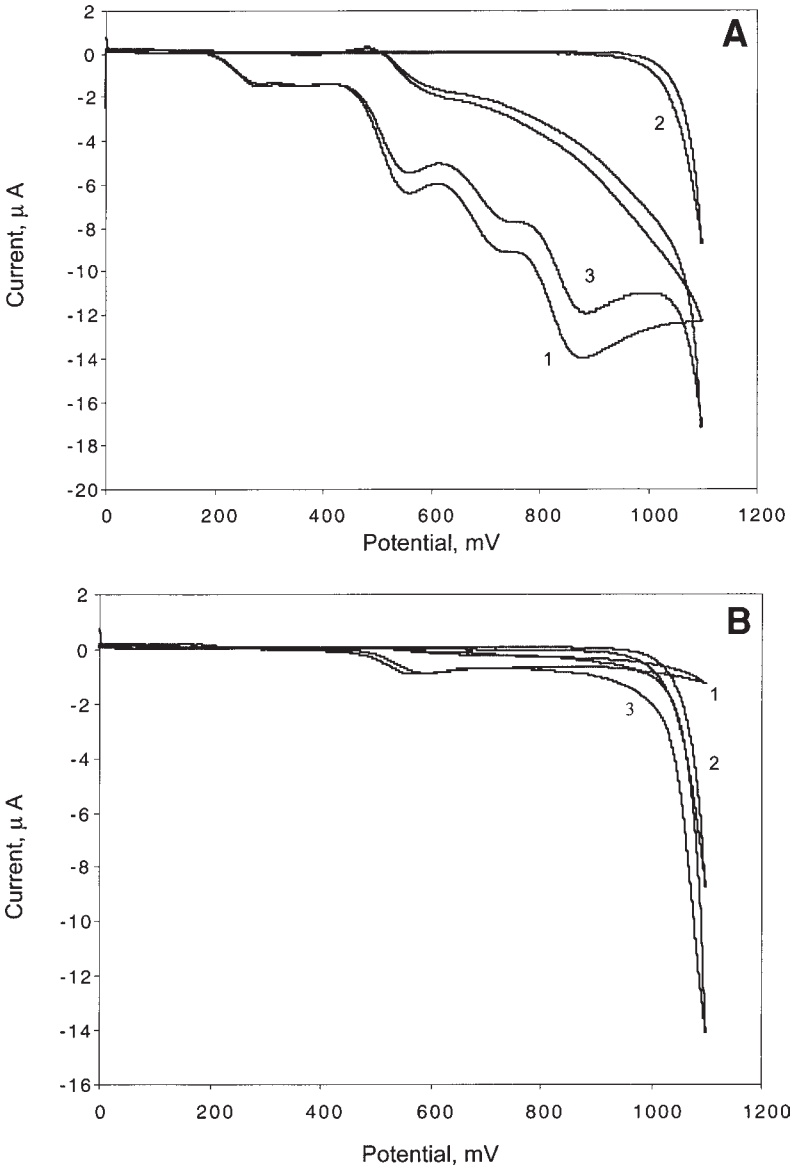


Fig. 4. Cyclic voltammograms of **(A)** PPNa or **(B)** SPP (0.2 m*M*) and VOH (2 m*M*) and their mixture, at a scan rate of 5 mV/s. The start potential was 0 mV. 1, mediator; 2, veratryl alcohol; 3, mediator and veratryl alcohol.

twofold (Fig. 4B). At higher potential (>1000 mV), the polarization curves for both compounds showed an increase in oxidation currents. It is likely that the increase in current originates from veratryl alcohol oxidation by the high redox potential intermediates formed at the electrode surface. These data are promising in terms of the possibility of veratryl alcohol oxidation by the intermediates formed in the course of SPP and PPNa electrooxidation.

Table 3
Oxidation of Veratryl Alcohol Mediated by Various Compounds[a]

Composition of reaction mixture	Degree of veratryl alcohol oxidation (%) after 48 h of incubation
Laccase + SPP + veratryl alcohol	35.0
Laccase + PPNa + veratryl alcohol	3.5
Laccase + ABTS + veratryl alcohol	75.7
SPP + veratryl alcohol	1.00
PPNa + veratryl alcohol	0.4
ABTS + veratryl alcohol	1.00
Laccase + veratryl alcohol	1.4

[a]Initial veratryl alcohol concentration = 10^{-3} *M*, concentration of mediator = 5×10^{-3} *M*, and concentration of laccase = 10^{-6} *M*.

An analysis of literature on xenobiotic degradation shows that the minimum redox potential of compounds used as mediators must be 450 mV. For example, it has been shown that the laccase/$K_4[Fe(CN)_6]$ system causes pulp bleaching and changes the kappa index *(16)*. However, for organic mediators, the required redox potential is higher, at least 600 mV *(6,15)*. Therefore, two PPNa oxidation products with redox potentials of 770 and 920 mV can be considered as candidates for the mediator role in the process of xenobiotic degradation, and in particular lignin biodegradation. Taking into account the effect of a substrate-to-enzyme ratio on the nature of intermediates formed in the course of PPNa oxidation, the optimization of conditions will play a critical role for the mediator. In the case of SPP, the laccase-catalyzed oxidation generates two intermediates with redox potentials sufficiently high (630 and 790 mV) to be applicable for biodegradation of lignin model compounds.

HPLC Studies on Veratryl Alcohol Oxidation in Laccase/Mediator System

To demonstrate the applicability of the selected compounds PPNa and SPP to *C. hirsutus* laccase-catalyzed degradation of the lignin model compound, veratryl alcohol, HPLC experiments were conducted (Table 3). The experiments were performed under conditions when a mediator is in sufficient excess over veratryl alcohol to generate a whole spectrum of oxidized mediator forms and, thus, to provide true nonenzymatic interaction between these oxidized forms and veratryl alcohol. The recently studied cooxidation of veratryl alcohol and ABTS *(29,30)* demonstrated that, at the mediator-to-substrate ratio of 0.15, veratryl alcohol is oxidized by laccase while ABTS activates the enzyme by reducing its "resting fully oxidized" state to the active form. Only at the mediator-to-substrate ratios of 2 and higher does true nonenzymatic interaction between the oxidized mediator and veratryl alcohol does occur *(29,30)*.

The control experiments in the absence of either enzyme or mediator show no veratryl alcohol degradation. The elution profile of veratryl alco-

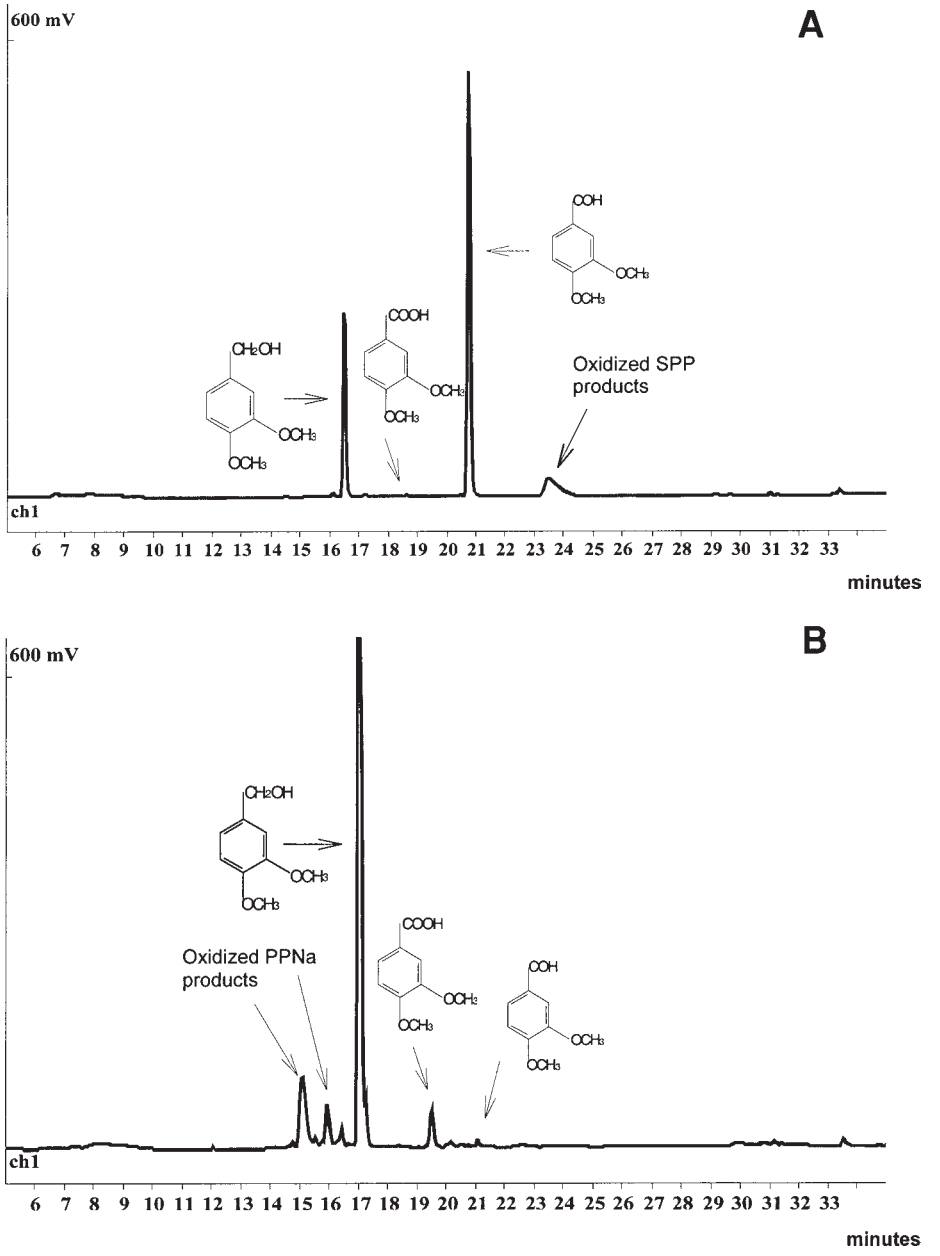


Fig. 5. Elution profile of HPLC-separated products of laccase-catalyzed degradation of veratryl alcohol in presence of **(A)** SPP and **(B)** PPNa (48-h incubation). Conditions are as given in Materials and Methods.

hol oxidation products obtained with SPP and PPNa as mediators is shown in Fig. 5A and Fig. 5B, respectively. In the case of SPP, the major product is represented by veratryl aldehyde, while in the case of PPNa veratric acid is also detected. It is widely accepted that veratryl aldehyde is the major

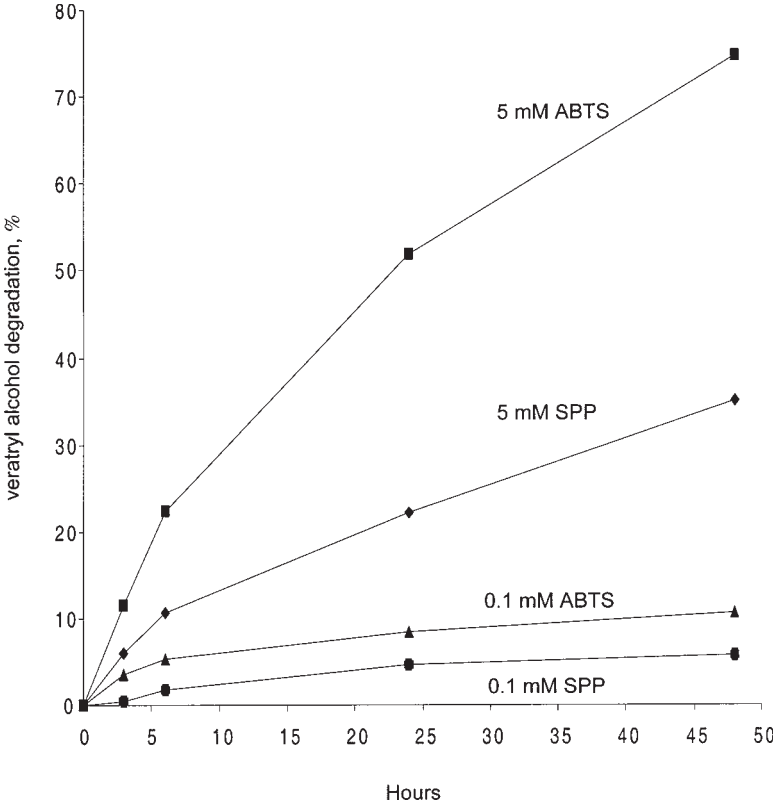


Fig. 6. Time course of veratryl alcohol (1 m*M*) oxidation catalyzed by *C. hirsutus* laccase at different mediator concentrations (ABTS or SPP). Conditions are given in Materials and Methods.

product of enzymatic degradation of veratryl alcohol, and detection is traditionally based only on these compounds *(6,7)*. Our experiments, for the first time, detected significant amounts of veratric acid as a product of further oxidation of veratryl aldehyde. Both profiles (Fig. 5A,B) demonstrate the presence of veratric acid in the eluate. Its content was much higher with PPNa used as a mediator (Fig. 5B), although the overall conversion was rather low. This observation agrees with the spectral and electrochemical data on PPNa. In the case of electrochemical oxidation of PPNa at low potential, no changes were observed on veratryl alcohol addition, while at high potentials an increase in current was recorded (Fig. 4). Two of the PPNa oxidation intermediates have redox potentials lower than 600 mV and are therefore insufficient to oxidize veratryl alcohol. On the other hand, two of the four PPNa oxidation intermediates have a very high redox potential (770 and 920 mV), which are sufficient to further oxidize model compounds, and in this particular case, veratryl alcohol to veratric acid.

The data presented in Tables 2 and 3, and the comparison of the time courses for veratryl alcohol oxidation with SPP and ABTS (Fig. 6), prove that mediator properties of SPP are comparable with those of ABTS.

The data show, however, that the newly discovered mediator is about 30–40% as efficient as ABTS under the same experimental conditions. The partial reversibility of the oxidation process observed in electrochemical experiments on SPP indicates poor stability of high-potential oxidation intermediates formed in the course of subsequent oxidation of SPP. This may be a reason for the incomplete degradation of veratryl alcohol in the enzyme/SPP system as compared with the enzyme/ABTS system, in which ABTS dication is known to be a stable oxidizing intermediate *(29,30)*. However, in the case of PPNa, its low effectiveness in comparison with ABTS originates from its poor K_m value (Table 2). PPNa concentration under the experimental conditions is comparable with the K_m value, and if PPNa oxidation intermediates are unstable and therefore incapable of cycling, the rate of its enzymatic oxidation will progressively decrease.

The cost of the new mediator is two to three orders of magnitude lower than that of ABTS, making it a very attractive candidate for commercial applications. Further work on construction and characterization of a series of novel mediators of the phenylpyrazolone class are ongoing in our laboratory. The work is of both practical interest and fundamental importance in terms of the elucidation of the structure-property relationships for radicals of organic compounds.

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