ISSN 0027-1314, Moscow University Chemistry Bulletin, 2008, Vol. 63, No. 2, pp. 94–98. © Allerton Press, Inc., 2008.
Original Russian Text © M.A. Gorbacheva, G.P. Shumakovich, O.V. Morozova, A.V. Strel'tsov, E.A. Zaitseva, S.V. Shleev, 2008, published in Vestnik Moskovskogo Universiteta. Khimiya, 2008, No. 2, pp. 117–121.

Comparative Study of Biocatalytic Reactions of High and Low Redox Potential Fungal and Plant Laccases in Homogeneous and Heterogeneous Reactions

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Received November 23, 2007

Abstract—The homogeneous catalytic and heterogeneous bioelectrocatalytic properties of high redox potential fungal laccases and low redox potential plant laccase have been compared. The fungal and plant laccases exhibit radically different catalytic activities as a function of pH with respect to substrates donating only electrons and substrates donating both hydrogen atoms and electrons, as well as in the bioelectrocatalytic reaction of dioxygen reduction. It is suggested that the difference between the biocatalytic properties of these enzymes correlates with their role in lignin metabolism.

DOI: 10.3103/S0027131408020107

Laccases (*p*-diphenol:dioxygen oxidoreductase, EC 1.10.3.2) belong to the class of blue oxidases and catalyze the oxidation of a large number of organic and inorganic substrates by dioxygen, which is thereby reduced to water (bypassing the stage of formation of hydrogen peroxide) [1, 2]. The active site of laccases has four copper ions, and their combined interaction couples the one-electron oxidation of donor substrates with the four-electron reduction of dioxygen. The copper ions have historically been divided into three types: T1 is a blue copper site, which has an absorption band at about 600 nm in electronic spectra and gives rise to an EPR spectrum with narrow hyperfine splitting; T2 is a mononuclear site, which has no intense features in the UV and visible spectrum but gives rise to a characteristic EPR spectrum; and T3 is a diamagnetic binuclear center, which has an absorption band at about 330 nm as a rather diffuse shoulder. The T2 and T3 sites form a trinuclear cluster, which is responsible for the reduction of dioxygen to water [3]. Depending on the redox potential of the T1 site, laccases are divided into high and low redox potential ones [4]. The mechanism of laccase catalysis is accepted to involve three stages: (1) reduction of the T1 copper ion by the donor substrate, (2) intramolecular electron transfer by the copper ions of the active site, and (3) reduction of dioxygen to water by the trinuclear copper cluster [2].

Laccases are widely encountered in fungi [5] and plants [6]. Laccase-like oxidases have also been found in microorganisms [7] and animals [2]. Plant laccases are involved in free-radical reactions of lignin synthesis, whereas fungal laccases have broader functions. They are involved in the morphogenesis of fungi, their protection from stress, and lignin degradation [8]. It is possible that the localization of laccases in the cell and the pH of a medium at which these enzymes function correlate with their physiological activity, which determines the set of substrates for them and the conditions of their functioning. The redox potential of the T1 site of laccases is of crucial importance for the manifestation of the substrate specificity of these enzymes.

This paper deals with a comparative study of biocatalytic reactions of the high redox potential fungal laccases of *Trametes hirsuta* and *Cerrena maxima* and the low redox potential laccase from the latex of the Japanese lacquer tree *Rhus vernicifera*.

EXPERIMENTAL

Microorganism culturing and enzyme purification. Producer strains of basidial fungi *T. hirsuta* and *C. maxima* were cultured as described in [9]. The following sequence of enzyme purification stages was used: precipitation of proteins from the culture broth by ammonium sulfate (0–90% saturation); low-pressure ion exchange chromatography on Sevacel DEAE 52 (Reanal, Hungary); adsorption chromatography on HTP Biogel (Bio-Rad, United States); rechromatography on DEAE-Toyopearl 650M (Toyo Soda, Japan). The final stage of purification of all enzymes was high-pressure liquid chromatography on a BioSep-SEC-S

2000 Phenomenex column (United States) with the use of the Stayer HPLC system (Aquilon, Russia). All stages of enzyme purification were carried out at 4°C.

The enzyme preparation from the latex of the Japanese lacquer tree *R. vernicifera* was courtesy of Prof. B. Reinhammar. The preparation was additionally purified by high-pressure chromatography. All enzyme preparations under consideration were homogeneous as probed by SDS electrophoresis.

Molecular weight determination. The molecular weight of laccases was determined by SDS polyacrylamide electrophoresis (SDS PAGE). The following marker proteins were used: cellulose (94.6 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), carboanhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). The protein concentration in solutions was determined as described in [10].

Kinetic studies. The activity of laccases in the reactions of oxidation of organic and inorganic substrates was determined spectrophotometrically from the formation rate of the product and electrochemically with the use of a Clark oxygen electrode by measuring the decrease in the reduction current of dioxygen consumed during the enzymatic reaction at a working electrode voltage of –600 mV. The kinetic parameters of the substrate oxidation reactions were calculated taking into account their stoichiometry.

The laccase-catalyzed enzymatic reactions were carried out in a 40 mM universal buffer solution at a specified pH.

Bioelectrocatalytic experiments were carried out on a CV-50W voltammetric analyzer (Bioanalytical Systems, United States) using a three electrode electrochemical cell. A spectroscopic graphite rod (Ringsdorff Werke, Germany) was used as a working electrode. Enzymes were immobilized on the surface of the working electrode by means of adsorption of a 10 μL sample of an enzyme preparation for 30 min. The unadsorbed enzyme was removed by repeated washing of the electrode with a working buffer solution.

Cyclic voltammograms were recorded for the renewed surface of the electrode at different pHs in the voltage range from –200 to 1000 mV against Ag/AgCl (210 mV versus the standard hydrogen electrode (vs. SHE)) at a potential sweep rate of 10 mV/s.

Reagents. The following reagents were used: boric acid, ABTS (diammonium 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (Sigma), hydroquinone (Roth), $K_4Fe(CN)_6 \cdot 3H_2O$ (Alfa Aesar), NaOH (Fluka), H_3PO_4, CH_3COOH, NaF, pyrocathechol (Russian State Standard, special purity grade), syringaldazine (ICN), and guaiacol (Acros Organics). All solutions, except those for fermentation, were prepared in deionized water obtained on a Milli Q water system (Millipore, United States).

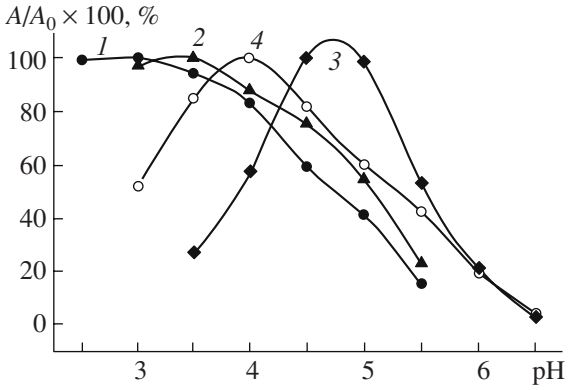

Fig. 1. pH Dependence of the reactions of oxidation of donors of electrons (*1*) ABTS (1 mM) and (*2*) $K_4Fe(CN)_6$ (10 mM) and donors of hydrogen atoms (*3*) syringaldazine (0.025 mM) and (*4*) hydroquinone (10 mM) in the presence of the laccase from *Trametes hirsuta* (0.8×10^{-8} M) in 0.04 M universal buffer.

RESULTS AND DISCUSSION

One approach to understanding the physiological role of fungal and plant laccases is determination of the activity of these enzymes with respect to different substrates at various pHs of a solution in both homogeneous and heterogeneous systems. The plots of the activity of high redox potential enzymes from basidial fungi *T. hirsuta* and *C. maxima* versus pH for different substrates are rather similar and differ only in shape for substrates donating only electrons and substrates donating both hydrogen atoms and electrons.

Figure 1 shows that high redox potential fungal laccase of basidial fungus *T. hirsuta* exhibits a catalytic activity up to pH 6.5 for both types of substrates. For electron-donating substrates (ABTS, $K_4Fe(CN)_6$), as pH increases, the enzyme activity first remains virtually unchanged and then monotonically decreases. For substrates donating hydrogen atoms (hydroquinone, syringaldazine), the plot of the activity of fungal laccases on pH is bell-shaped. The difference in the pH dependences for laccase substrates of different donor types can be explained as follows: For both types of substrates, when the pH of a solution is varied to alkaline values, the initial rate of enzymatic reactions decreases due to their inhibition by hydroxyl ions, which bind to the trinuclear T2/T3 clusters of laccases [2]. For the phenolic organic substrates (donors of hydrogen atoms) of fungal laccases, the ionization potential decreases with an increase in the pH of a solution and, hence, the reaction rate increases. A combination of these two processes is responsible for the bell-shaped curves of the pH dependence of the enzymatic reaction for substrates donors of hydrogen atoms. For electron-donating

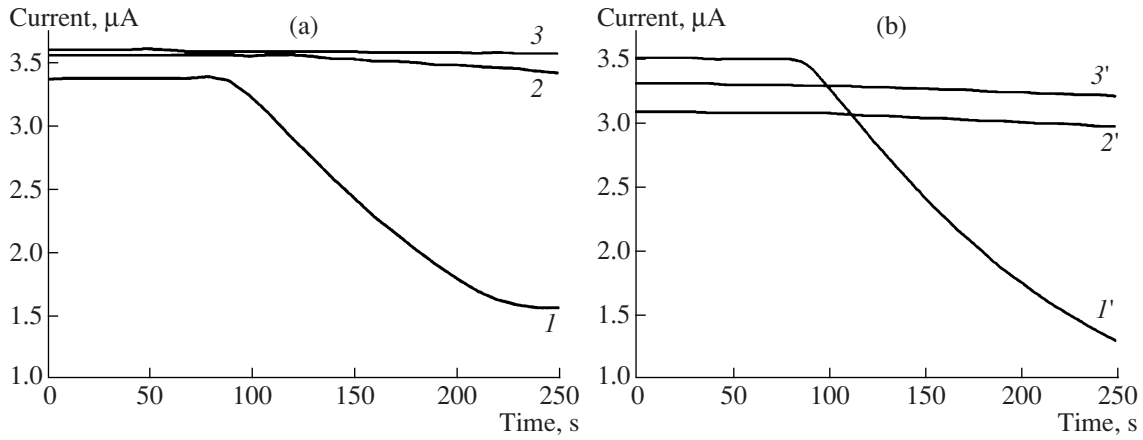

Fig. 2. Inhibition of the initial rate of oxidation of (a) ABTS (1 mM) and (b) hydroquinone (10 mM) catalyzed by the laccase from *Cerrena maxima* (3.3×10^{-8} M) in 0.04 M universal buffer at pH 4: (*1*, *1*') in the absence of an inhibitor, (*2*, *2*') in the presence of 1 mM NaF, and (*3*, *3*') the background in the absence of the laccase.

ABTS and potassium hexacyanoferrate, the substrate ionization potential does not respond to a change in pH, so that the plot of the pH dependence shows a small plateau and then a smooth decrease in the substrate oxidation reaction rate.

Additional evidence of the above features of fungal laccases is the inhibition of the catalytic oxidation reactions of both types of substrates by fluoride ions (Figs. 2a and 2b). Fluoride ions, like hydroxyl ions, bind to the trinuclear T2/T3 cluster and disrupt intramolecular electron transfer from the enzyme substrate to dioxygen. The inhibition of the enzymatic oxidation reactions of ABTS and hydroquinone by 1 mM NaF is 95 and 96%, respectively.

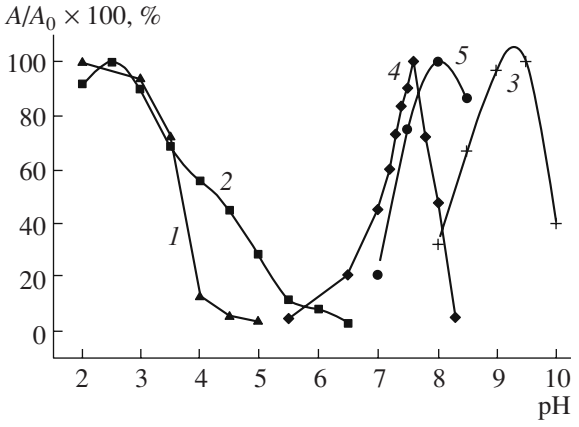

Fig. 3. pH dependence of the reactions of oxidation of donors of electrons (*1*) ABTS (1 mM) and (*2*) $K_4Fe(CN)_6$ (10 mM) and donors of hydrogen atoms (*3*) guaiacol (10 mM), (*4*) hydroquinone (10 mM), and (*5*) pyrocathechol (10 mM) in the presence of the laccase from *Rhus vernicifera* (9.2×10^{-8} M) in 0.04 M universal buffer.

A different situation is observed upon the enzymatic oxidation of the substrates of both groups in the presence of the low redox potential laccase from the Japanese lacquer tree *R. vernicifera*. As distinct from high redox potential fungal laccases *T. hirsuta* and *C. maxima*, low redox potential plant laccase is able to catalyze the oxidation of organic substrates donors of hydrogen atoms in neutral and weakly alkaline solutions and remains catalytically inactive at acidic pHs. At the same time, this enzyme is catalytically active with respect to electron-donating substrates only in the acidic pH range (Fig. 3). This means that, for substrates donating hydrogen atoms, this enzyme is poorly inhibited by hydroxyl ions. As distinct from high redox potential fungal laccases, the activity of low redox potential plant laccase is poorly inhibited by fluoride ions in the neutral and alkaline range when hydrogen-donating substrates are used. However, like high redox potential enzymes from basidial fungi, *R. vernicifera* laccase loses its activity in the presence of halide ions during oxidation of electron-donor substrates in acid solutions.

Comparison of the pH dependences of high redox potential fungal laccases and low redox potential plant laccase makes it possible to elucidate their physiological function, in particular, their role in lignin degradation or synthesis. The high redox potential laccases from wood-decaying white-rot fungi *T. hirsuta* and *C. maxima* have the redox potentials 780 ± 20 and 750 ± 20 mV vs. SHE [4, 11], respectively, whereas the laccase from the latex of the Japanese lacquer tree *R. vernicifera* is a low redox potential laccase and has the redox potential 440 ± 20 mV vs. SHE [4, 12].

Different lignocellulose materials contain micro amounts of manganese. The manganese ions Mn^{3+} are strong oxidants and can directly oxidize nonphenolic

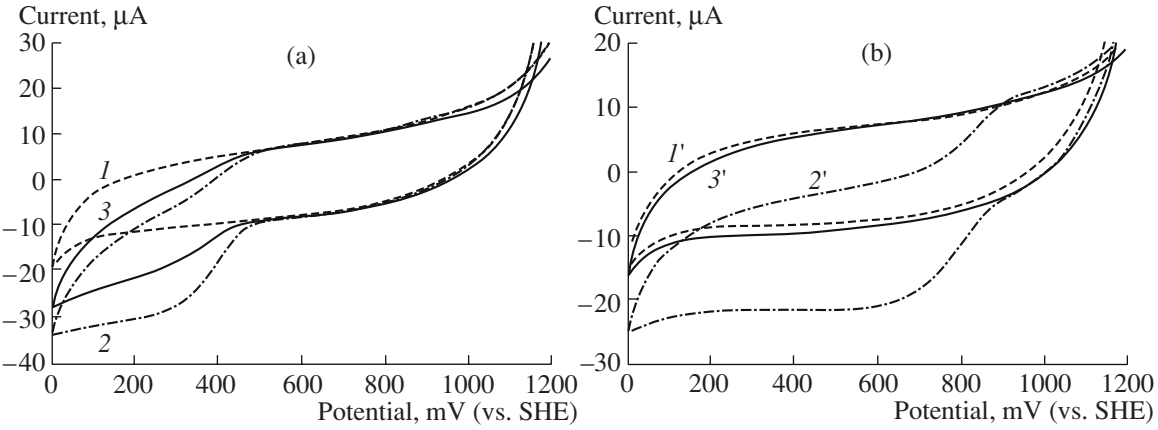

Fig. 4. Cyclic voltammograms of the electrooxidation of dioxygen on graphite electrodes modified with (a) low redox potential laccase from *Rhus vernicifera* and (b) high redox potential fungal laccase from *Cerrena maxima* in 0.04 M universal buffer at pH (a) 7.5) and (b) 4.0: (*1*, *1*') the background reaction in the absence of the enzyme, (*2*, *2*') in the presence of the adsorbed enzyme, and (*3*, *3*') in the presence of the enzyme and inhibitor.

lignin substructures, thus promoting lignin degradation. It was previously shown [13] that Mn^{3+} ions are generated upon enzymatic oxidation of Mn^{2+} ions in the presence of chelating agents (oxalic, tartaric, and fumaric acids). The latter are necessary for reducing the redox potential of the Mn^{2+}/Mn^{3+} pair [14], thus making possible the oxidation of Mn^{2+} by dioxygen in the presence of high redox potential laccases. In particular, the potential of the beginning of the oxidation of the Mn^{2+}–tartrate complex to the Mn^{3+} tartrate complex is about 950 mV, so that it is thermodynamically possible for the former to be oxidized by dioxygen in the presence of high redox potential laccases from white-rot fungi, which have redox potentials in the range 750–800 mV [4, 11–13]. Thus, high redox potential laccases can be directly involved in lignin degradation, not only through direct oxidation of the phenolic lignin substructures, but also through indirect oxidation of the nonphenolic lignin substructures by the triply charged manganese ions that form in the course of the enzymatic reaction catalyzed by fungal laccases. The low redox potential laccase from *R. vernicifera*, with a redox potential of 440 mV, is unable to catalyze this reaction and, therefore, cannot be involved in lignin degradation. However, in the range of neutral and weakly alkaline pHs, the lacquer tree laccase efficiently catalyzes the oxidation of guaiacol, hydroquinone, and dimethoxyphenol, which are structural units of lignin. Thus, due to free-radical reactions, this enzyme can be involved in the synthesis of lignin from monomers.

Bioelectrocatalytic studies. The laccases under consideration were immobilized by means of physical adsorption on the surface of a graphite electrode, which is, in this case, an electron-donor substrate in the reaction of dioxygen electroreduction. Both enzymes catalyze the electroreduction of dioxygen to water. The dioxygen reduction half-wave potential is close to the redox potential of the T1 copper ions of the high and low redox potential enzymes under consideration (Fig. 4).

Like the homogeneous reaction catalyzed by the low redox potential laccase from the Japanese lacquer tree, the biocatalytic reaction of dioxygen electroreduction in the presence of this enzyme is inhibited by fluoride ions, almost completely in acid solutions and poorly in neutral solutions (Fig. 4a). The activity of the *C. maxima* laccase is completely inhibited by fluoride ions in acid solutions (Fig. 4b). In addition, fungal laccases are inactive in neutral and weakly alkaline solutions.

ACKNOWLEDGMENTS

We are grateful to A.V. Sof'in for his help with the purification of the enzyme preparations.

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