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Mediator-Assisted Laccase-Catalyzed Oxidation of 4-Hydroxybiphenyl

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Abstract—The kinetics of oxidation of 4-hydroxybiphenyl (4-HBP) catalyzed by laccase from *Polyporus pinsitus* was studied in the presence of methyl syringate (MS), which acts as an electron-transfer mediator. Measurements were performed in 0.05 M acetate buffer, pH 5.5, in the presence of 4-HBP, MS, and laccase. It is shown that the oxidation rate of the lowly reactive substrate 4-HBP significantly increases during synergistic action of the highly reactive substrate MS. Bimolecular kinetic constants of interaction between the oxidized form of laccase and MS, the former and 4-HBP, and the oxidized form of MS and 4-HBP were calculated. A kinetic scheme of the synergistic substrate action is suggested; based on this scheme, the dependence of the initial rate on reagent concentration is derived. Analyzing experimental data, we obtained kinetic constants close to those obtained by modeling the processes.

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As a result of rapid development of chemical industry in the second half of the XX century, compounds used in households and industry are synthesized on a large scale. Many stable organic preparations are based on thermally stable and water-insoluble biphenyl—a conjugated compound able to donate electrons. Biphenyl can be easily halogenated or nitrated, and it is also able to participate in other electrophilic exchange reactions. As a result of chlorination, polychlorinated biphenyls, which are stable organic pollutants, are formed.

Hydroxylation of biphenyl results in formation of hydroxybiphenyl (HBP); the latter was one of ten most widely used pesticide ingredients as early as 1995 and ranks second in its use as a pesticide in agriculture [1]. The para position in the biphenyl molecule is the most subject to primary hydroxylation [2, 3].

Long use of these compounds, their resistance to degradation, and their lipophilicity has resulted in global environmental pollution. Conventional disposal methods (burning and burying) are expensive and not always efficient, because for such compounds the half-life period is from three weeks to two years in air and more than six years in soil.

Recent scientific developments are aimed at biodegradation of these pollutants; this method is not expensive and not environmentally harmful [4]. Most studies on biphenyl biodegradation are based on the use of various bacterial groups [5-8]. Aerobic bacteria have been found that can enzymatically degrade lowly chlorinated biphenyl compounds [9, 10].

By gene engineering, now it is possible to use recombinant enzyme forms for recovery of organic pollutants. Such enzymes function within a wide range of pH, temperature, and substrate concentrations. To increase efficiency of catalytic oxidation of biphenyls, various mediators (2,2′-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid, 1-hydroxybenzotriazole) and thermally stable laccase isolated from AN 28-2 *Trametes* fungi were added to the reaction mixture, and also organic solvents (water–organic systems) were used for preparation of reagents [4].

Laccase (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2) belongs to a group of copper-containing pro-

Abbreviations: 4-HBP) 4-hydroxybiphenyl; MS) methyl syringate.

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teins, catalyzes oxidation of phenol derivatives (*o*- and *p*-diphenols, aminophenols, polyphenols, and polyamines), organic polymers (lignin and humic acids), and also some inorganic ions via one-electron transfer with accompanying reduction of molecular oxygen to water. It is known that laccase contains four copper ions in different surroundings: one of TI type (one-electron transfer), one of TII type (reoxidation of TI-type Cu, stabilization of H_2O_2 intermediate), and two of TIII type (four-electron transfer on O_2 with formation of H_2O) [11]. The data indicate that for most oxidized substrates, the catalytic effect (k_{cat}/K_m) probably depends on the redox potential of TI-type Cu [12-14]. As a result of the presence of redox mediators in the reaction mixture, the number of laccase-oxidized substrates increases [15, 16].

Laccase-mediatory systems are used, for example, in sawdust delignification [17] and in development of biosensors [18] and biofuel elements [19]. Oxidation of substrates by laccase-mediatory systems proceeds without direct contact between substrate and enzyme but by the action of the oxidized form of the mediator. Efficiency of laccase-mediatory systems is due not only to a rather high redox potential of TI-type Cu (0.5-0.8 V versus the standard hydrogen electrode) [13], but also by a relatively high standard redox potential of the mediator [20].

In this paper we present data on oxidation of 4-hydroxybiphenyl (4-HBP) by recombinant laccase from *Polyporus pinsitus*. The effect of the mediator (methyl syringate) on the initial rate of catalytic oxidation of 4-HBP was studied. A kinetic scheme of the process and mathematical model of dependence of the initial rate on substrate concentrations are presented, and calculated kinetic parameters are reported.

MATERIALS AND METHODS

In this study, we used *P. pinsitus* recombinant laccase from Novozymes A/S (Denmark) without additional purification, 4-HBP from Aldrich (Germany), and acetic acid and sodium acetate of chemically pure grade from Reakhim (Russia). Methyl syringate from Lancaster Synthesis (USA) was additionally purified by recrystallization from ethanol. Laccase concentration was determined spectrophotometrically using an Ultrospec II UV/Visible spectrophotometer from LKB (Sweden) and taking molar extinction $\varepsilon_{280} = 7.8 \cdot 10^2$ $M^{-1} \cdot cm^{-1}$ [21].

Oxidation of 4-HBP was studied in a thermostatted quartz cuvette with optical path length of 1 cm, using a Hitachi MPF-4 spectrofluorimeter (Japan). Fluorescence intensity of 4-HBP was measured at $\lambda_{ex} = 270$ nm and $\lambda_{em} = 330$ nm. Kinetics were measured in 0.05 M acetate buffer, pH 5.5, at 25°C at the following concentrations: $(1\text{-}10) \cdot 10^{-6}$ M 4-HBP, $(2\text{-}100) \cdot 10^{-6}$ M MS, and $(6\text{-}100) \cdot 10^{-9}$ M laccase. The reaction was initiated by addition of the enzyme solution. Fluorescence intensity was calibrated using 4-HBP solutions of various concentrations at the same pH. While studying the effect of MS on the rate of 4-HBP oxidation, decrease in 4-HBP fluorescence intensity was noted. This is due to partial optical absorption by MS: the latter has absorption maximum at 280 nm. So changes in the substrate fluorescence intensity were normalized, taking the initial value of fluorescence intensity as the value of 4-HBP concentration. To calculate the initial rate of 4-HBP oxidation (V_0), we used the initial (linear) segments of kinetic curves.

To explain laccase-catalyzed oxidation of 4-HBP and MS mediatory effect on this process, we suggest the following equations:

$$E_{red} + O_2 + 4H^+ \rightarrow E_{ox} + 2H_2O \quad k_1, \qquad (1)$$

$$E_{ox} + M_{red} \rightarrow E_{red} + M_{ox} + 4H^+ \quad k_2, \qquad (2)$$

$$E_{ox} + S \rightarrow E_{red} + S_{ox} \quad k_3, \qquad (3)$$

$$M_{ox} + S + H^+ \rightarrow M_{ox} + S_{ox} \quad k_4, \qquad (4)$$

where S, M_{red} and M_{ox}, and E_{red} and E_{ox} are concentrations of 4-HBP and reduced and oxidized forms of mediator and enzyme, respectively; O_2 concentration is constant ($2.54 \cdot 10^{-4}$ M), and k_1, k_2, k_3, k_4 are the rate constants in Eqs. (1)-(4), respectively.

In the presence of mediator, the dependence of the initial rate of oxidation of 4-HBP on its concentration can be expressed by Eqs. (1)-(4) using the symbolic processor of the MathCAD 2001 Prof program and taking the steady-state conditions in relation to enzyme and mediator concentrations.

Under non-steady-state conditions this process was modeled by solution of a system of differential equations characterizing change in reagent concentrations of reactions (1)-(4), using the KinFitSim 2.0 program [22]. The reaction rate was calculated as change in 4-HBP concentration during 44-45 sec.

To process the data, we used the MathCAD 2001 Prof, KinFitSim 2.0, and GraFit 3.01 programs.

RESULTS

In acetate buffer, pH 5.5, at 25°C, the initial rate V_0 of 4-HBP oxidation linearly increased in the range $(0.4\text{-}11) \cdot 10^{-9}$ M/sec on increase in enzyme concentration from $6 \cdot 10^{-9}$ to 10^{-7} M. When $5 \cdot 10^{-6}$ M methyl syringate was added to the reaction mixture, V_0 increased from $8 \cdot 10^{-9}$ to $8.8 \cdot 10^{-8}$ M/sec (Fig. 1). Dependence of V_0 on enzyme concentration in the presence of MS is represented by a hyperbolic curve.

The initial rate of 4-HBP oxidation linearly increased at low laccase concentrations ($(1\text{-}30) \cdot 10^{-9}$ M). Negligible V_0 change was observed on increase in enzyme

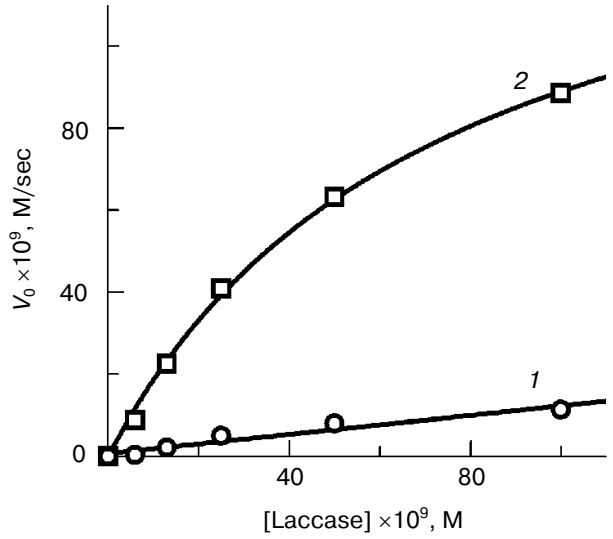

Fig. 1. Initial rate of 4-HBP oxidation versus laccase concentration in the absence (*1*) and in the presence (*2*) of MS. Reaction conditions: 0.05 M acetate buffer, pH 5.5, $6 \cdot 10^{-6}$ M 4-HBP, $5 \cdot 10^{-6}$ M MS, 25°C. Experimental points of (*1*) were approximated by a linear equation (correlation coefficient $R = 0.9670$). Experimental points of (*2*) were approximated by a hyperbolic curve using the Michaelis–Menten equation ($V_{max} = (1.5 \pm 0.1) \cdot 10^{-8}$ M/sec and $K_m = (7 \pm 1) \cdot 10^{-9}$ M).

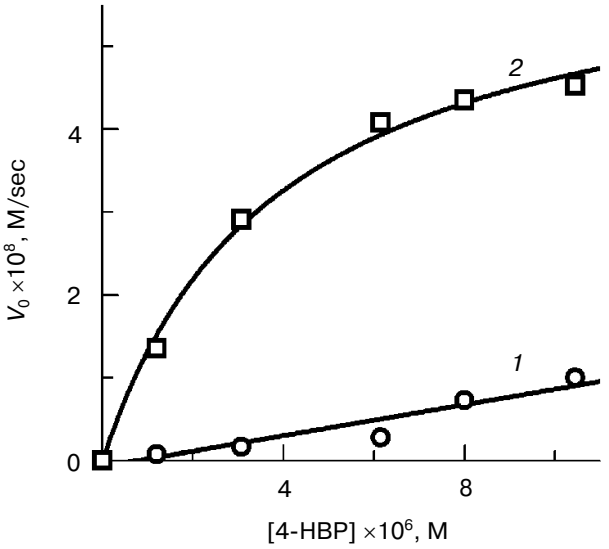

Fig. 2. Initial rate of laccase-catalyzed oxidation versus 4-HBP concentration in the absence (*1*) and in the presence (*2*) of MS. Reaction conditions: 0.05 M acetate buffer, pH 5.5, $2 \cdot 10^{-8}$ M laccase, $5 \cdot 10^{-6}$ M MS, 25°C. Experimental points of (*1*) were approximated by a linear equation (correlation coefficient $R = 0.9547$). Experimental points of (*2*) were approximated by a hyperbolic curve using the Michaelis–Menten equation ($V_{max} = (6.4 \pm 0.4) \cdot 10^{-8}$ M/sec, $K_m = (3.8 \pm 0.7) \cdot 10^{-6}$ M).

concentration in the range $(5\text{-}10) \cdot 10^{-8}$ M. The data suggest that non-enzymatic interaction reaction between MS radical and 4-HBP is a limiting stage of the process (4). The bimolecular constant k_4 was calculated from approximation of V_0 dependence on laccase concentration in the presence of MS as $V_{max}/([4\text{-HBP}][MS])$ and was equal to $(5.0 \pm 0.8) \cdot 10^3$ $M^{-1} \cdot sec^{-1}$. V_0 linearly depended on 4-HBP concentration in the range $(1\text{-}10.5) \cdot 10^{-6}$ M and was equal to $(0.8\text{-}10) \cdot 10^{-9}$ M/sec (Fig. 2).

Bimolecular constant k_3 was calculated from the ratio of the slope of a line approximating the dependence on laccase concentration and was equal to $(4.7 \pm 0.5) \cdot 10^4$ $M^{-1} \cdot sec^{-1}$. In the presence of MS, the initial rate of substrate oxidation changed in the range $(1.4\text{-}4.5) \cdot 10^{-8}$ M/sec. The data indicate that the presence of MS in the reaction mixture results in 15-17-fold increase in the initial reaction rate at relatively low 4-HBP concentrations (less than K_m). On increase in 4-HBP concentration, the initial rate of oxidation increased negligibly. This fact suggests that in the presence of MS, the initial rate of 4-HBP oxidation is defined by the rates of 4-HBP enzymatic oxidation (3) and chemical interaction of the oxidized MS with 4-HBP (4). At laccase concentration $2 \cdot 10^{-8}$ M, the total rate of the process is not completely limited by the chemical reaction. The apparent rate constant $k_4 = (3.4 \pm 0.7) \cdot 10^3$ $M^{-1} \cdot sec^{-1}$ calculated from the data presented in Fig. 2 is less than a constant calculated at high laccase concentrations.

A plot of V_0 versus MS concentration is presented in Fig. 3. At MS concentrations in the range from $2 \cdot 10^{-6}$ to $5 \cdot 10^{-5}$ M, the dependence is saturated and is described by a hyperbolic function in accordance with the Michaelis–Menten equation. Apparent kinetic parameters are $V_{max} = 110.7 \pm 6.8$ nM/sec and $K_m = 8.9 \pm 1.9$ μM.

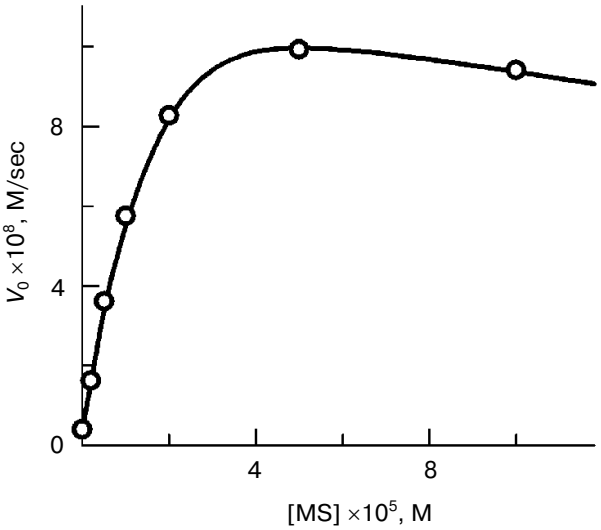

Fig. 3. Initial rate of laccase-catalyzed 4-HBP oxidation versus MS concentration. Reaction conditions: 0.05 M acetate buffer, pH 5.5, $2 \cdot 10^{-8}$ M laccase, $6 \cdot 10^{-6}$ M 4-HBP, 25°C. A curve plotted through the experimental points is an approximation obtained by modeling of non-steady-state kinetics with parameters: $k_1 = 10^6$ $M^{-1} \cdot sec^{-1}$, $k_2 = 5 \cdot 10^5$ $M^{-1} \cdot sec^{-1}$, $k_3 = 3.8 \cdot 10^4$ $M^{-1} \cdot sec^{-1}$, $k_4 = 5.5 \cdot 10^3$ $M^{-1} \cdot sec^{-1}$. Concentrations: $2.54 \cdot 10^{-4}$ M O_2, $6 \cdot 10^{-5}$ M 4-HBP, $2 \cdot 10^{-8}$ M E_t.

Kinetic characteristics of laccase-catalyzed oxidation of 4-hydroxybiphenyl in the presence of methyl syringate in 0.05 M acetate buffer, pH 5.5, at 25°C

Method of determination	k_1, $M^{-1}\cdot sec^{-1}$	k_2, $M^{-1}\cdot sec^{-1}$	k_3, $M^{-1}\cdot sec^{-1}$	k_4, $M^{-1}\cdot sec^{-1}$
Experimental data	10^6	$(6.0 \pm 2.0) \cdot 10^5$	$(4.7 \pm 0.5) \cdot 10^4$	$(5.0 \pm 0.8) \cdot 10^3$
Mathematical modeling	10^6	$(4.7 \pm 0.5) \cdot 10^5$	$(3.3 \pm 0.5) \cdot 10^4$	$(4.4 \pm 0.8) \cdot 10^3$

Note: The value of k_1 was taken from [27].

In this case, the enzymatic stage of the process and bimolecular constant k_2 characterizing efficiency of interaction between MS and enzyme and calculated as $V_{max}/(K_m\cdot[\text{laccase}]) = (6.0 \pm 2.0)\cdot 10^5$ $M^{-1}\cdot sec^{-1}$ are limiting. Bimolecular kinetic constants calculated from the experimental data are presented in the table.

DISCUSSION

Enzymes—biological catalysts—are highly active and specific. Laccase belongs to oxidoreductase family and exhibits wide substrate specificity. Using redox mediators, it is possible to extend the spectrum of laccase-oxidized substrates. Efficiencies of enzyme–mediator systems have been shown [15, 20, 23, 24]. The considered process of laccase-catalyzed oxidation of 4-HBP in the presence of MS belongs to synergistic reaction type. When a reaction proceeds via such a mechanism, methyl syringate as well as the enzyme is distributed between two forms—oxidized and reduced:

$$M_t = M_{(ox)} + M_{(red)},$$

$$E_t = E_{(ox)} + E_{(red)}.$$

To explain the laccase-catalyzed oxidation of 4-HBP and MS mediatory effect on this process, we used Eqs. (1)-(4); according to these equations, one-electron oxidation of 4-HBP occurs simultaneously with four-electron reduction of molecular oxygen. When MS is included into the enzymatic process, transport of an electron from mediator to enzyme is accompanied by electron transfer from a 4-HBP molecule to the oxidized mediator; this results in regeneration of the mediator (Eqs. (3) and (4)). The dependence of initial rate of oxidation of 4-HBP on its concentration in the presence of a mediator is defined by Eqs. (1)-(4), using the steady-state conditions for enzyme and mediator ((5)-(7)):

$$V = S\,(k_3 E_{ox} + k_4 M_{ox}), \quad (5)$$

$$M_{ox} = M_t\, k_2 E_{ox}/(k_2 E_{ox} + k_4 S), \quad (6)$$

$$E_{ox} = 2 E_t k_1 O_2 k_4 S [k_4 S k_1 O_2 - k_2 E_t k_1 O_2 + k_4 S^2 k_3 + S M_t k_4 k_2 + (k_2^2 E_t^2 k_1^2 O_2^2 + 2 k_2 E_t k_1^2 O_2^2 k_4 S + 2 k_2 E_t k_1 O_2 k_4 S^2 k_3 - 2 k_2^2 E_t k_1 O_2 S M_t k_4 + k_4^2 S^2 k_1^2 O_2^2 + 2 k_4^2 S^3 k_1 O_2 k_3 + 2 k_4^2 S^2 k_1 O_2 M_t k_2 + k_4^2 S^4 k_3^2 + 2 k_4^2 S^3 k_3 M_t k_2 + S^2 M_t^2 k_4^2 k_2^2)^{1/2}]^{-1}, \quad (7)$$

where S, E_t, O_2, and M_t are initial concentrations of 4-HBP, laccase, oxygen, and MS, respectively.

The kinetics of the process was mathematically modeled at the following concentrations: $2\cdot 10^{-8}$ M laccase, $2.54\cdot 10^{-4}$ M oxidant (O_2), $5\cdot 10^{-6}$ M MS, and $6\cdot 10^{-6}$ M 4-HBP; steady state was also suggested. The results indicate that quasi-steady state for laccase is established during 10^{-2} sec, and the initial rate of 4-HBP oxidation is directly proportional to its concentration in the range $(1\text{-}30)\cdot 10^{-9}$ M (Fig. 1).

Mediator concentration change was modeled under analogous conditions; the results indicate that for MS, the quasi-steady state is not established even after 180 sec. However, if the initial MS concentration is less than 10^{-6} M, the quasi-steady state is established during 50-60 sec [25].

Kinetic constants obtained by mathematical modeling using Eqs. (1)-(4) and by solution of Eqs. (5)-(7) are close in value to those obtained experimentally (table). This suggests that the considered process really proceeds via the suggested scheme (Eqs. (1)-(4)).

A synergistic mechanism was considered earlier, but excess substrate concentration in relation to mediator concentration was one of the necessary conditions for the reaction proceeding via such a mechanism [26]. The 4-HBP and MS concentrations used in this study and the values of kinetic constant obtained experimentally do not suggest quasi-steady state in relation to mediator. Due to this, the derivation of equation for the initial rate of the

process is not completely correct, and the latter should be determined using conditions of non-steady-state kinetics. At MS concentrations higher than $5 \cdot 10^{-5}$ M, decrease in the initial rate is observed. Under these conditions, quasi-steady state is not established; this allows considerations of the dependence of the initial rate in the studied range of MS concentrations under non-steady-state conditions (Fig. 3). Nonetheless, for 4-HBP the initial rate can be derived only if the steady-state process is suggested. In this case, the steady-state condition is provided at the cost of high substrate/mediator concentration ratio at low MS concentrations.

So, the kinetics of laccase-catalyzed oxidation of 4-HBP in the presence of MS functioning as a redox-active mediator was studied. The suggested kinetic scheme of synergistic oxidation of lowly active 4-HBP in the presence of MS and a mathematical model of this process were experimentally proved. It was shown that depending on the experimental conditions (the ratio of enzyme, substrate, and mediator concentrations), the process can proceed under steady-state as well as non-steady-state conditions in relation to the oxidized mediator concentration. The results are of interest for further search for enzyme–mediator systems, which not only enlarge the spectrum of enzyme-oxidized substrates but also increase degradation efficiency of slowly oxidized phenol compounds.

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