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Non-Enzymatic Interaction of Reaction Products and Substrates in Peroxidase Catalysis

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Abstract—A quantitative approach for estimation of the non-enzymatic interaction between ammonium 2,2′-azino-*bis*(3-ethylbenzthiazoline-6-sulfonate) (ABTS) oxidation product and a poorly oxidized substrate was developed using a system including tobacco peroxidase, a mediator substrate (ABTS), and a second substrate. The approach is based on the establishment of a pseudo-steady-state concentration of the ABTS oxidation product in the course of co-oxidation with a poor substrate. A mathematical description of the experimental curve shape has been proposed to linearize the kinetic data and estimate the rate constant for such non-enzymatic interaction. The rate constants calculated from the steady-state kinetics for the non-enzymatic interaction of ABTS oxidation product with phenol and resorcinol were 360 ± 40 and 770 ± 60 $M^{-1} \cdot sec^{-1}$, respectively. The values obtained have the same order of magnitude as the rate constant for ABTS oxidation product interaction with veratryl alcohol, calculated from electrochemical measurements (170 $M^{-1} \cdot sec^{-1}$) by Donal Leech's group. However, the kinetic curves for co-oxidation of ABTS and veratryl alcohol catalyzed by tobacco peroxidase exhibit a pronounced lag-period, which either points to the high rate of the non-enzymatic interaction between ABTS oxidation product and veratryl alcohol and thus, contradicts the electrochemical calculations, or indicates an enzymatic nature of the co-oxidation phenomenon in this particular case.

Key words: tobacco peroxidase, mediator, second-order rate constant, phenol, resorcinol, veratryl alcohol

Peroxidase (EC 1.11.1.7) catalyzes the oxidation of electron donors of various chemical structure with hydrogen peroxide. If not accounting for the formation of Michaelis-type complexes [1], the formal description of the peroxidase catalytic mechanism is represented by the system of chemical reactions as shown in Scheme 1:

$$E + H_2O_2 \xrightarrow{k_1} EI + H_2O,$$

$$EI + S \xrightarrow{k_2} EII + P,$$

$$EII + S \xrightarrow{k_3} E + H_2O + P,$$

Scheme 1

where E, EI, and EII are the native enzyme and its Compounds I and II, respectively, and S and P are a substrate and the product of its one-electron oxidation.

The Michaelis complexes are usually omitted because they cannot be detected for native peroxidases by pre-steady-state methods, although these complexes have been observed for some mutant forms [2, 3].

The phenomenon of substrate–substrate activation is well known in peroxidase catalysis [4, 5]. This type of activation manifests itself by the stimulated oxidation of a poorly or non-oxidized ("bad") substrate (S_1) in the presence of an easily oxidized ("good") substrate (S_2). Three different mechanisms can describe substrate–substrate activation. In the first case, Compound I is active towards a "bad" substrate, while Compound II does not oxidize it (Scheme 2):

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$$E + H_2O_2 \rightarrow EI + H_2O,$$

$$EI + S_1 \rightarrow EII + P_1,$$

$$EI + S_2 \rightarrow EII + P_2,$$

$$EII + S_1 \Leftrightarrow EII{-}S_1,$$

$$EII{-}S_1 \rightarrow E + H_2O + P_1.$$

Scheme 2

In this case, a "good" substrate is necessary to convert Compound II back to the resting enzyme form [6]. This mechanism stimulates the oxidation of a "bad" substrate and inhibits the oxidation of a "good" one. Formally, a "bad" substrate behaves as a competitive inhibitor with respect to a "good" one. The productivity of the "bad" substrate binding has to be proved independently.

In the second case, the oxidation of a "good" substrate cannot be detected before the consumption of a "bad" substrate is complete. The oxidation curves of a "good" substrate demonstrate lag-periods, whose duration is proportional to the concentration of the "bad" substrate. The molecular mechanism of this stimulation can be either enzymatic or non-enzymatic. If a "bad" substrate is oxidized in the course of its interaction with the complex of an enzyme and an enzyme-bound oxidized "good" substrate [7-11], one can claim the enzymatic nature of the stimulation mechanism (Scheme 3):

$$EI + VA \rightarrow EII{-}VA^{+\bullet},$$

$$EII{-}VA^{+\bullet} + S \rightarrow EII{-}VA + P,$$

$$EII{-}VA^{+\bullet} + VA \rightarrow EII{-}VA{-}VA^{+\bullet} \rightarrow E + VA + Vald,$$

Scheme 3

where EII-$VA^{+\bullet}$ is lignin peroxidase Compound II complex with veratryl alcohol cation radical, VAld is veratryl aldehyde, S_2 is a "bad" substrate, and P_2 is the product of its oxidation.

The formation of a complex between lignin peroxidase Compound II and veratryl alcohol cation radical has been proved experimentally [12-14]. The inability of veratryl alcohol cation radical to oxidize some substrates in the absence of the enzyme was interpreted as the higher redox potential of the enzyme-bound cation radical than its free form in solution [15].

The third case is represented by direct non-enzymatic interaction of an oxidized "good" substrate with a "bad" substrate (Scheme 4):

$$E_{red} \xrightarrow{[O_2] \text{ or } [H_2O_2]} E_{ox},$$

$$E_{ox} + A \rightarrow E_{red} + A^{+\bullet},$$

$$E_{ox} + A^{+\bullet} \rightarrow E_{red} + A^{2+},$$

$$2A^{2+} + VA \rightarrow 2A^{+\bullet} + Vald,$$

Scheme 4

where E_{red} and E_{ox} are reduced and oxidized form of an enzyme (laccase or peroxidase), A, $A^{+\bullet}$, A^{2+} are ammonium or sodium 2,2′-azino-*bis*(3-ethylbenzthiazoline-6-sulfonate) (ABTS) and its cation radical and dication, respectively.

This particular scheme of substrate co-oxidation is of great practical interest for the purpose of development of biotechnological methods of bleaching and lignin depolymerization in the paper industry. This type of interaction was proposed for the system consisting of laccase (copper-containing oxidase catalyzing the oxidation of various organic compounds with molecular dioxygen), lignin, and ABTS [16]. Electrochemically, ABTS is first oxidized to cation radical, $ABTS^{+\bullet}$, and then to dication, $ABTS^{2+}$ [16]. It has been proposed that it is the dication that mediates the oxidation of veratryl alcohol and lignin. The value of the rate constant for the interaction between veratryl alcohol and $ABTS^{2+}$, 170 $M^{-1}\cdot sec^{-1}$, has been calculated from electrochemical experiments in the absence of the enzyme [16].

The goal of the present work was to develop a quantitative approach for the evaluation of the mediator efficiency of a defined substrate using a model system including tobacco peroxidase and its poorly oxidized substrates, phenol and resorcinol. One of the best mediators known so far, ABTS, has been chosen as a "good" substrate. The developed method allows one to calculate the rate constants for the interaction of ABTS oxidation products with phenol or resorcinol from the steady-state kinetic data.

MATERIALS AND METHODS

The following reagents were used: ammonium 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), sodium acetate, sodium citrate, citric acid, phenol, resorcinol, veratryl alcohol from Sigma (USA). Tobacco peroxidase was isolated from transgenic plants as described in [17]. Enzyme concentration was determined from its absorbance at 403 nm using the molar extinction coefficient 108 $mM^{-1}\cdot cm^{-1}$ [18].

Peroxidase activity was assayed on a Shimadzu UV 120-02 spectrophotometer (Japan) at 25°C in 0.1 M Na-acetate or 0.05 M citrate-phosphate buffer (pH 4.5). The

extinction coefficient for ABTS oxidation product at 405 nm was taken as 36.8 $mM^{-1}\cdot cm^{-1}$ [19]. Hydrogen peroxide concentration was calculated from its absorbance (ε_{240} = 43.6 $M^{-1}\cdot cm^{-1}$) [20]. The determination of values of catalytic constants, K_m and V_{max}, were performed at a fixed hydrogen peroxide concentration (1.6 mM) and varied ABTS concentrations in the range of 10-500 μM.

RESULTS AND DISCUSSION

In this work the co-oxidation of ABTS and phenol (or ABTS and resorcinol) catalyzed by anionic tobacco peroxidase has been studied. Tobacco peroxidase was isolated in this laboratory and exhibits high stability in acidic medium [18, 21], and almost no activity towards phenol and iodide-anion [17]. Such behavior is likely to be a result of the presence of the negatively charged residue at the entrance to the enzyme active site protecting the heme from dissociation at extreme pH and restricting the access of substrates to the active site pocket [21]. Compact folding of the enzyme molecule provides a high activity towards the substrates which are oxidized through the electron transport chain such as ABTS.

The analysis of kinetic curves of ABTS oxidation in the presence of phenol (Fig. 1) reveals a drop in the ABTS conversion and a plateau corresponding to a steady-state concentration of ABTS oxidation product. The conversion degree decreases linearly with rising concentrations of phenol while the initial rates of ABTS oxidation remain unchanged within the experimental error (Figs. 1 and 2).

The data have been interpreted in the context of the third mechanism, i.e., non-enzymatic phenol oxidation with ABTS cation radical (Scheme 5):

$$E + H_2O_2 \xrightarrow{k_1} EI + H_2O,$$

$$EI + S \xrightarrow{k_2} EII + P,$$

$$EII + S \xrightarrow{k_3} E + P,$$

$$P + Ph \xrightarrow{k_{Ph}} S + Ph_{ox},$$

Scheme 5

where Ph and Ph_{ox} are phenol and its oxidation product.

The estimation of the rate constant for the non-enzymatic interaction between phenol and ABTS oxidation products was based on the mechanism of peroxidase catalysis. A steady-state kinetic approach cannot give the values of individual rate constants for the reduction of each oxidized compound of peroxidase (k_2 and k_3) presented in Schemes 1 and 5. The rate constant for Compound I reduction (k_2) is usually an order higher than that for Compound II reduction (k_3), and both constants are lower

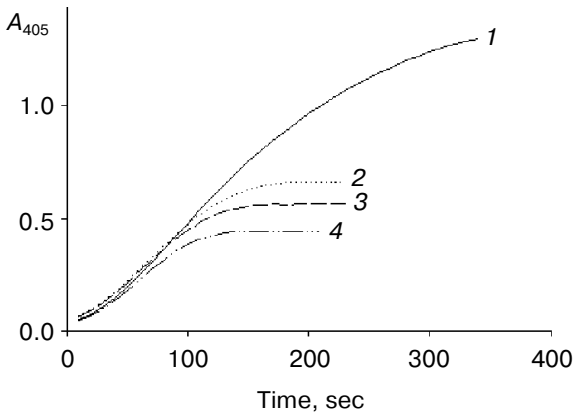

Fig. 1. Effect of phenol on ABTS oxidation catalyzed by 1 nM tobacco peroxidase in the absence (*1*) and in the presence of 12.5 (*2*), 25 (*3*), and 50 μM (*4*) phenol, respectively. Experimental conditions: 0.05 M citrate-phosphate buffer, pH 4.5, 40 μM ABTS, 1.6 mM hydrogen peroxide, 25°C.

than that for the enzyme oxidation with hydrogen peroxide (k_1) for most peroxidases. Thus, the rate-limiting step in peroxidase catalysis is the last one in the peroxidase cycle with rate constant k_3. Assuming $k_2 >> k_3$, the rate equation is presented in the form $E_0/v = 1/k_1[H_2O_2] + 1/k_3[S]$ and corresponds to the ping-pong type mechanism. For this mechanism, the concept of Michaelis constant is not fully correct because the rate constants in the Michaelis–Menten equation include substrate concentrations. For example, the constants for a donor substrate $v =$

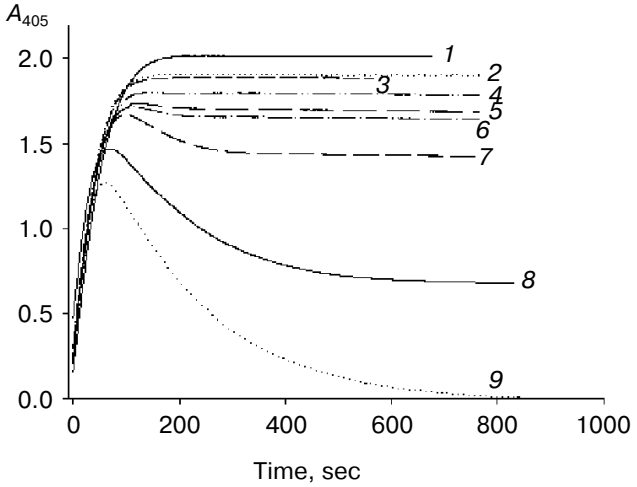

Fig. 2. Effect of phenol on kinetic curves of enzymatic oxidation of ABTS at 5 nM enzyme. The phenol concentration increases as follows: 0 (*1*), 2.44 (*2*), 4.9 (*3*), 7.3 (*4*), 9.8 (*5*), 12.2 (*6*), 24.2 (*7*), 48.8 (*8*), and 122 μM (*9*). Experimental conditions: 0.05 M citrate-phosphate buffer, pH 4.5, 60 μM ABTS, 1.6 mM hydrogen peroxide, 25°C.

$k_{cat}E_0[S]/(K_m + [S])$, where $K_m = k_1[H_2O_2]/k_3$ and $k_{cat} = 2k_1[H_2O_2]$, include the concentration of peroxide, and *vice versa*, the constants for hydrogen peroxide include the concentration of a donor substrate. Nevertheless, the modern biochemical literature widely uses both equations. Therefore, the classic Michaelis–Menten equation has been used to simplify the mathematical procedures.

In the absence of phenol at a fixed saturating concentration of hydrogen peroxide, the rate equation can be presented in the Michaelis–Menten form with S and P substituting for ABTS and its oxidation product:

$$d[P]/dt = k_{cat}[E]_0 [S] / (K_m + [S]). \quad (1)$$

Under the experimental conditions presented in Fig. 1, the concentration of ABTS was much lower than its apparent Michaelis constant of 0.15 mM. This allows one to further simplify the equation:

$$d[P]/dt = k_{cat}[E]_0 ([S]_0 - [P]) / K_m . \quad (2)$$

Accounting for the substrate material balance,

$$[P] + [S] = [S]_0 , \quad (3)$$

Eq. (2) can be written as:

$$d[P]/([S]_0 - [P]) = k_{cat} [E]_0 dt / K_m . \quad (4)$$

Integration results in:

$$[P] = [S]_0 \{1 - \exp [-(k_{cat}[E]_0 t / K_m)]\}. \quad (5)$$

Thus, in the absence of phenol the ABTS oxidation curve is an exponential with a saturation plateau corresponding to the initial ABTS concentration. This is the case experimentally observed in the work. (Fig. 1, curve *1*). In the presence of phenol (Ph), an additional reaction of its interaction with ABTS oxidation product occurs. Under the conditions of [ABTS] << K_m:

$$d[P]/dt = [E]_0[S]k_{cat}/K_m - k_{Ph} [P][Ph], \quad (6)$$

and taking into account that $[S] = [S]_0 - [P]$, we have:

$$d[P]/dt = [E]_0([S]_0 - [P]) k_{cat}/K_m - k_{Ph} [P][Ph]. \quad (7)$$

Assuming the phenol concentration is unchanged, Eq. (7) easily converts into:

$$[P] = k_{cat}/K_m [S]_0[E]_0 \{1 - \exp(-k_{cat} [E]_0 t/K_m - $$

$$- k_{Ph}[Ph]t)\}/\{(k_{cat}/K_m) [E]_0 + k_{Ph}[Ph]\}. \quad (8)$$

Therefore, at low ABTS concentration in the presence of phenol, kinetic curves of its oxidation will reach a steady-state plateau, and an increase in the phenol concentration will result in a drop in the substrate conversion.

The results presented in Fig. 1 were obtained at 1 nM tobacco peroxidase. In this case the rate of ABTS oxidation is not high and, given a low rate of non-enzymatic interaction of ABTS radical with phenol (see below), one can ignore phenol consumption in the course of the reaction recording. Relatively high concentrations of phenol do not principally change the shape of kinetic curves.

Figure 2 illustrates the effect of a higher enzyme concentration, which reveals the initial burst of ABTS product formation, which is especially sharp in the presence of high phenol concentrations. The obtained dependence indicates the non-enzymatic reaction of phenol and ABTS oxidation product interaction to be rate-limiting in the discussed system.

The kinetic curve of ABTS oxidation in the presence of phenol presents a curve reaching a steady-state plateau with the conversion degree decreasing proportional to the phenol concentration. The curve fits to Eq. (8), which gives the expression for the experimentally determined steady-state concentration of ABTS oxidation product in the presence of phenol as follows:

$$[P]_{lim}(Ph) = k_{cat}/K_m[S]_0[E]_0/\{(k_{cat}/K_m)[E]_0 + k_{Ph}[Ph]\}, \quad (9)$$

and in the absence of phenol as $[P]_{lim}(0) = [S]_0$ (see Eq. (9)). The inversed conversion degree $[P]_{lim}(0)/[P]_{lim}(Ph)$ can be easily calculated from the experimental curves and linearly depends on the phenol concentration:

$$[P]_{lim}(0)/[P]_{lim}(Ph) = 1 + k_{Ph}K_m[Ph]/k_{cat}[E]_0. \quad (10)$$

The equation predicts a linear plot in the $[P]_{lim}(0)/[P]_{lim}(Ph)$ ~ [Ph] coordinates passing through the point (0; 1), which is actually observed (Fig. 3). Given

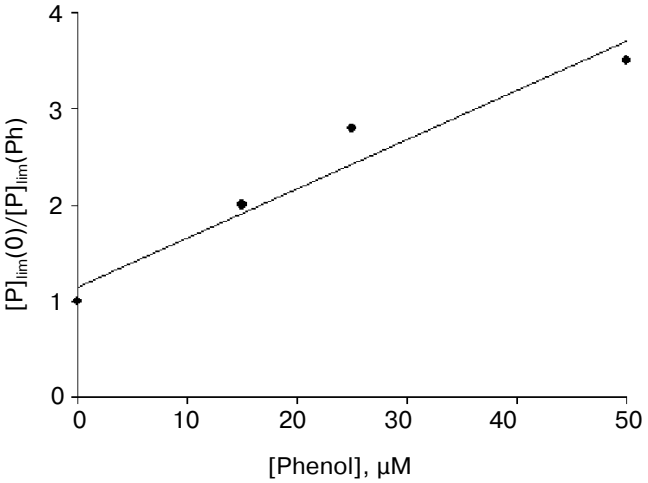

Fig. 3. Linearization of the data in Fig. 1 in accordance with Eq. (10).

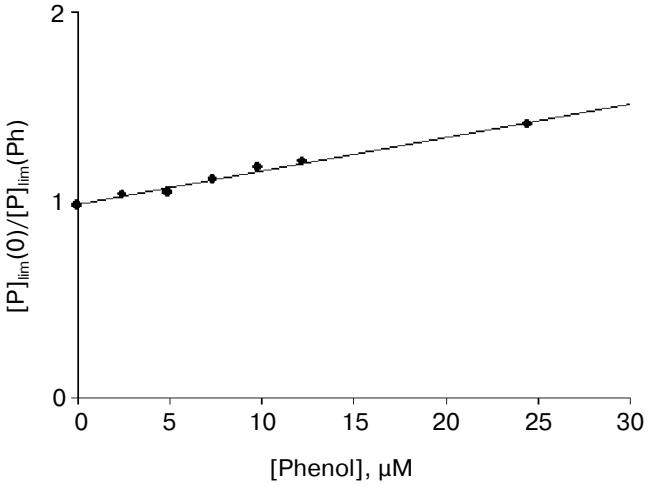

Fig. 4. Linearization of the data in Fig. 2 in accordance with Eq. (10).

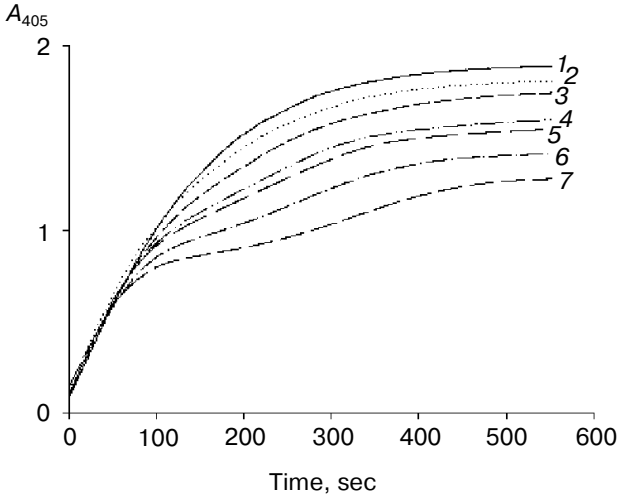

Fig. 5. Co-oxidation of resorcinol and ABTS in the presence of 5 nM tobacco peroxidase. The resorcinol concentration increases as follows: 0 (*1*), 1 (*2*), 2 (*3*), 3.5 (*4*), 5 (*5*), 6.1 (*6*), and 10 μM (*7*). Experimental conditions as in Fig. 2.

the value of $K_m/k_{cat}[E]_0 = K_m/V_{max}$ for the particular experimental conditions, the apparent rate constant for the phenol and ABTS oxidation product interaction, k_{Ph}, can be determined from the slope of the above dependence equal to $k_{Ph}K_m/k_{cat}[E]_0$. At 1 nM tobacco peroxidase (Fig. 1), the value of k_{Ph} was calculated as 360 ± 40 $M^{-1}\cdot sec^{-1}$ (Fig. 3). At the higher enzyme concentration (5 nM) (Fig. 2), the apparent rate constant for the phenol and ABTS oxidation product interaction was 300 ± 30 $M^{-1}\cdot sec^{-1}$ (Fig. 4), which agrees with the previous determination within the experimental error.

A similar picture is observed for the enzymatic co-oxidation of resorcinol and ABTS (Fig. 5). The rate constant for the interaction of the ABTS cation-radical with resorcinol is determined as 770 ± 60 $M^{-1}\cdot sec^{-1}$ (Fig. 6).

If veratryl alcohol, which is oxidized by tobacco peroxidase with a 2-3 order lower efficiency compared to ABTS [18], is used as a "bad" substrate, the picture is dramatically different (Fig. 7), i.e., a lag-period whose duration is linearly proportional to the concentration of veratryl alcohol is observed. If the lag-period corresponds to the non-enzymatic interaction between veratryl alcohol and ABTS oxidation product, its rate constant should be at the level of diffusion-controlled rate constants ($>10^8$ $M^{-1}\cdot sec^{-1}$). This conclusion disagrees with the value determined from electrochemical experiments as

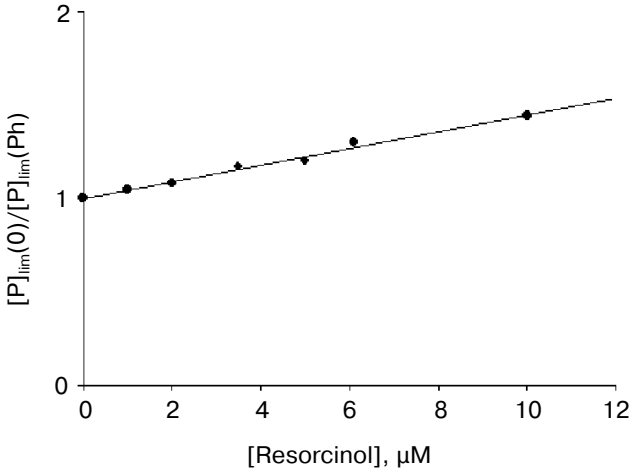

Fig. 6. Linearization of the data in Fig. 5 in accordance with Eq. (10).

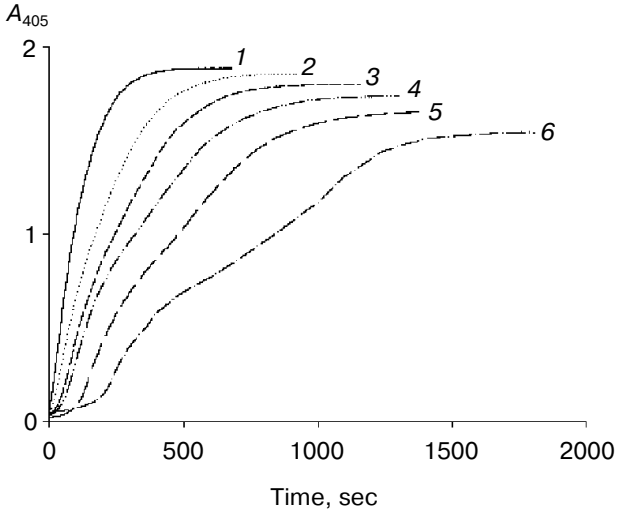

Fig. 7. Co-oxidation of veratryl alcohol and ABTS catalyzed by tobacco peroxidase (5 nM) in the absence (*1*) and in the presence of 1.9 (*2*), 3.1 (*3*), 4.7 (*4*), 6.3 (*5*), and 9.5 μM (*6*) veratryl alcohol, respectively. Experimental conditions as in Fig. 2.

170 $M^{-1}\cdot sec^{-1}$ [16]. We suppose that either the value of the rate constant determined from the electrochemical data is not correct, or the oxidation of veratryl alcohol proceeds through the mechanism presented in Scheme 3 via its interaction with the complex enzyme–$ABTS^{+\cdot}$. If the latter case holds for veratryl alcohol, the question arises why the same scheme is not working for phenol and resorcinol. The answer for this question requires new experiments on co-oxidation of ABTS and veratryl alcohol catalyzed by various types of laccase and peroxidase aimed to evaluate the effect of the nature of the enzyme on the observed kinetic dependences.

The present work proposes a mathematical description for the experimentally observed phenomenon of co-oxidation of two peroxidase substrates assuming non-enzymatic interaction between a poorly oxidized substrate and the oxidation product of a substrate-mediator. The developed approach allows quantitative evaluation of the ABTS mediator properties with respect to poorly oxidized peroxidase substrates.

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