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Oxidation of Phenolic Compounds by Peroxidase in the Presence of Soluble Polymers

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Abstract—The kinetics of *Coprinus cinereus* peroxidase-catalyzed 1-naphthol, 2-naphthol, and 4-hydroxybiphenyl oxidation was investigated. The initial rates of the naphthols' and 4-hydroxybiphenyl oxidations were linearly dependent on enzyme concentration. The rates depended on substrate concentration and saturated at concentrations above 100 μM of hydrogen peroxide, 25-50 μM of naphthols, and 10 μM of 4-hydroxybiphenyl. At the peroxide concentration 100 μM calculated K_m and the maximal rate (V_{max}) were 74.7 μM and 0.53 μM/sec or 175 μM and 2.0 μM/sec for 1- or 2-naphthol, respectively, and 29.68 μM and 0.42 μM/sec for 4-hydroxybiphenyl. Kinetic measurements of exhaustive naphthol and 4-hydroxybiphenyl oxidation showed that peroxidase is inactivated during the oxidation of the substrates. Different factors and additives, water soluble polymers and albumins (PEG, PEI, PL, BSA, HSA), influenced the initial naphthols and 4-hydroxybiphenyl oxidation rates, peroxidase inactivation rates, and the degree of the substrate conversion. Addition of albumin increased turnover number of naphthols oxidation 1.5-4 times. Light scattering increase was observed when peroxidase-catalyzed oxidation reaction was investigated and suggested that insoluble particles were formed during the process. The addition of polymers, change of concentration and ionic strength of the solution as well as the number of other factors influenced the observed light scattering. The number of particles formed during peroxidase-catalyzed naphthols' and 4-hydroxybiphenyl oxidation and their distribution according to size in the interval 2.5-300 μm were detected by particle counting in solutions.

Key words: fungal peroxidase, naphthol, 4-hydroxybiphenyl, albumin, polyethylene glycol, polyethyleneimine, poly-L-lysine, turnover number, light scattering, microparticles

Phenolic compounds are found in most industrial wastewaters, for example, from wood and polymer processing, textile and dye industries, etc. Most of these phenolic compounds are toxic and classified as pollutants, dangerous for human and animal health [1]. The majority of these substances are carcinogens. As times passes, their cumulative concentration in nature can reach critical levels [2]. Phenolic compounds in wastewaters are now processed by different techniques: solvent extraction [3, 4], degradation by microorganisms [5, 6], adsorption on activated carbon [7, 8], or electrochemical oxidation [9, 10]. These techniques are inefficient and costly. Often, the final result includes the formation of even more toxic side-products [11]. Introduction of enzymes opens the way to more efficient handling of a broad spectrum of phenolic compounds. The enzymes can be used over wide ranges of pH, temperature, and substrate concentrations. A method for oxidation of aromatic compounds by peroxidase was first proposed by Klibanov et al. [12].

The problem of catalytic oxidation of phenols occupies scientists around the world, where peroxidase extracted from plant sources (soybean, horseradish) is most often used [13, 14]. The extraction of these enzymes is a laborious and expensive process. In recent years, recombinant peroxidase forms produced by gene engineering techniques are increasingly used in studies. These enzymes are more available than and as efficient as their wild-type analogs.

Peroxidase (EC 1.11.1.7) is a heme-containing glycoprotein catalyzing oxidative, peroxidative, and oxygenative oxidation of substrates. It is characterized by high specificity toward the hydrogen acceptor substrate (which is hydrogen peroxide) and low specificity toward hydrogen donor substrate. The number of these substrates is

Abbreviations: PEG) polyethylene glycol; PEI) polyethyleneimine; PL) poly-L-lysine; PO) peroxidase; BSA) bovine serum albumin; HSA) human serum albumin; AMB) N,N′-dimethylamine-4-(4-morpholine)benzene.

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high; among those are such phenolic compounds as naphthol, 4-hydroxybiphenyl, hydroquinone, guaiacol, etc.

Enzymes are inactivated during the catalytic process of oxidation of phenolic compounds. The mechanism of enzyme inactivation is not completely known. There are some theories about the mechanism of enzyme inactivation. According to one of them, radicals are formed during the substrate oxidation by peroxidase. They react spontaneously, forming insoluble polymers, which contribute to enzyme inactivation. Another mechanism is implemented via the formation of microparticles during the reaction, where enzyme molecules are adsorbed. The presence of a diffusion layer around the particles prevents substrate molecules from accessing the enzyme active site, which contributes to the inhibition of the reaction.

The goal of this work was to investigate the oxidation kinetics of 1- and 2-naphthols and 4-hydroxybiphenyl by peroxidase, as well as light scattering intensity during this process, the influence of water-soluble polymers, and to study proposed inactivation mechanisms [15, 16].

MATERIALS AND METHODS

The following reagents were used in this work: recombinant peroxidase (PO) from *Coprinus cinereus* fungus (Novozymes A/S, Denmark); 30% hydrogen peroxide solution, sodium acetate, acetic acid, potassium hydrophosphate, and 4-hydroxybiphenyl (Reakhim, Russia); 1- and 2-naphthol (Aldrich, USA); polyethyleneimine (PEI, M_r 30,000-40,000), poly-L-lysine (PL) (M_r 6000-9000, n = 40-60), bovine serum albumin (BSA, 99%), human serum albumin (HSA, 98%) (Serva, Germany); polyethylene glycol (PEG) (M_r 40,000) (Ferak, Germany). N,N′-Dimethylamine-4-(4-morpholine)benzene (AMB) was synthesized according to [17].

Concentrations of PO, hydrogen peroxide, HSA, and BSA were determined spectrophotometrically using molar absorption coefficients ε_{405} = 108 $mM^{-1}\cdot cm^{-1}$ [18], ε_{240} = 39.4 $M^{-1}\cdot cm^{-1}$ [19], ε_{280} = 36.6 $mM^{-1}\cdot cm^{-1}$, and ε_{280} = 43.6 $mM^{-1}\cdot cm^{-1}$ [20] for each reagent, accordingly.

Kinetic measurements were performed in a thermostatted quartz cuvette using a computerized LKB Ultrospec II LKB UV/Visible spectrophotometer and MPF-4 spectrofluorimeter (Hitachi, Japan). Fluorescence intensity was measured at excitation λ = 320 and 328 nm for 1- and 2-naphthol, respectively, and emission (fluorescence) λ = 460 nm; excitation λ = 270 nm and emission λ = 330 nm for 4-hydroxybiphenyl. Light scattering intensity was measured at 500 and 400 nm for naphthols and 4-hydroxybiphenyl, respectively.

The number of particles and their size-distribution were determined using a system for particle counting in liquids (Hiac/Royco 9703, USA). The 16-channel system was programmed for measuring number of particles and their size-distribution in the 2.5-300 μm range.

Kinetic measurements were performed in 50 mM acetate buffer at pH 5.5 and 25°C, naphthol concentrations of 25 μM, 4-hydroxybiphenyl concentration of 10 μM, and hydrogen peroxide concentration of 100 μM. The reaction was started by adding PO solution. The fluorescence was calibrated using the substrate solutions at the same pH. Initial reaction rate (v_0) was determined from the fluorescence kinetic curves for the substrates, using initial linear ranges of the kinetic curves for the calculation of v_0 of peroxidation. To analyze the dependence of v_0 on the substrate concentration and determine the kinetic parameters V_{max} and K_m of the reactions, the Michaelis–Menten equation was used.

The peroxidase oxidation of the investigated substrates is characterized by a ping-pong mechanism and includes two bimolecular stages of substrate interaction with an enzyme active site (bb-mechanism). The peroxidase interacts with H_2O_2 (reduces it via two-electron transfer) forming a CI complex. Naphthol and 4-hydroxybiphenyl (electron donors) react with the formed complex (thus peroxidase reduction takes place) and are oxidized forming a product.

The following scheme was used to explain the substrate oxidation by peroxidase:

$$E + H_2O_2 \rightarrow CI + H_2O \quad (k_{ox}), \tag{1}$$

$$CI + \text{substrate} \rightarrow E + \text{product} \quad (k_{red}), \tag{2}$$

$$E + \text{product} \rightarrow E_{in} \quad (k_{in}), \tag{3}$$

$$CI + \text{product} \rightarrow E_{in} \quad (k_{in}), \tag{4}$$

$$\text{Product} + \text{product} \rightarrow \text{polymer} \quad (k_p), \tag{5}$$

where E_{in} is inactive form of the enzyme, and CI is peroxidase complex.

According to this scheme the initial reaction rate is [21]:

$$v_0 = k_{ox}k_{red}[H_2O_2][S][E]/(k_{ox}[H_2O_2] + k_{red}[S]), \tag{6}$$

where [S], $[H_2O_2]$, and [E] are initial concentrations of the substrate, hydrogen peroxide, and peroxidase.

Kinetic parameters (k_{ox}, k_{red}) of naphthol and 4-hydroxybiphenyl oxidation catalyzed by peroxidase and also the enzyme inactivation constant (k_{in}) were calculated using Runge–Kutta adaptive method for solving the system of differential equations presented by the kinetic system. Computer programs GraFit 3.01 and Mathcad PLUS 5.0 were employed for data processing.

Determination of catalytic turnover number (TN) for peroxidase. Naphthol, hydrogen peroxide, and PO were incubated in a 10 ml reactor. After selected periods of time, aliquots of 2 ml of the analyzed solution were taken, and enzyme activity and naphthol concentration were measured. Peroxidase activity was measured spectropho-

tometrically at 604 nm based on AMB cation-radical formation rate (AMB concentration was 50 μM, hydrogen peroxide concentration was 100 μM). Concurrently, the fluorescence intensity was measured, and naphthol concentration was determined according to the calibration curve. From the ratio between the amount of oxidized naphthol and concentration of enzyme active form, it could be determined what amount of substrate oxidized 1 mol of the enzyme until the complete inactivation. The enzyme turnover number was calculated based on the expression [16]:

$$TN = (N_0 - N)/(E_0 - E), \quad (7)$$

where $(N_0 - N)/(E_0 - E)$ is tangent of the strait dependence line inclination of oxidized naphthol amount on inactivated enzyme amount.

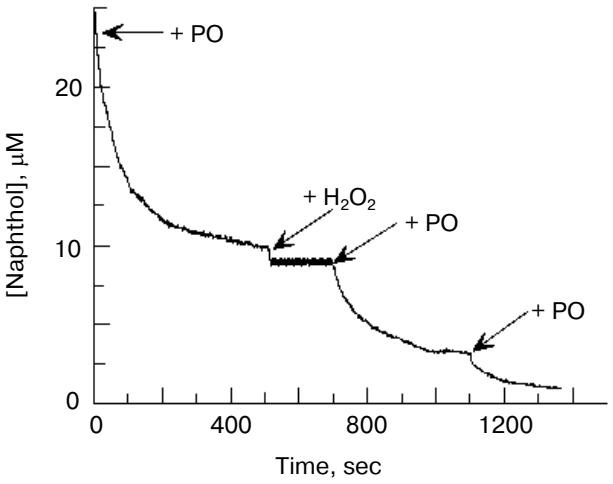

Fig. 2. Influence of additions on the peroxidase-catalyzed oxidation of naphthol. Conditions the same as in Fig. 1.

RESULTS

Kinetics of naphthol and 4-hydroxybiphenyl oxidation by peroxidase. The initial rate of 1-naphthol oxidation in the presence of hydrogen peroxide is directly proportional to PO concentration in the 0.016-2.6 nM range and equals 0.016-0.48 μM/sec (acetate buffer, pH 5.5, 25°C). In the case of 2-naphthol the oxidation rate was 3 times higher under the same conditions. The dependency of v_0 on initial substrate concentration (Fig. 1) is described by the Michaelis–Menten equation. The obtained parameters for 1-naphthol, 2-naphthol, and 4-hydroxybiphenyl are: V_{max} = 0.53, 2.0, and 0.42 μM/sec; K_m = 74.7, 175, and 29.7 μM, respectively. The dependences of v_0 on hydrogen peroxide concentration were obtained at fixed concentrations of naphthol and 4-hydroxybiphenyl. At peroxide concentrations higher than 50 μM, substrate saturation is observed. The obtained kinetic parameters are: V_{max} = 0.16, 0.45, and 0.09 μM/sec, and K_m = 33.6, 80.72, and 6.34 μM for 1-naphthol, 2-naphthol, and 4-hydroxybiphenyl, respectively. Comprehensive studies revealed that during the peroxidase oxidation, the kinetic curve reaches its saturation, but the complete conversion of the investigated substrates is not observed. Introduction of an additional amount of H_2O_2 did not affect the reaction. On the other hand, the introduction of an additional portion of enzyme caused further naphthol oxidation (Fig. 2). A similar pattern was observed in the case of 4-hydroxybiphenyl. This confirms the assumption that peroxidase is being inactivated during the reaction.

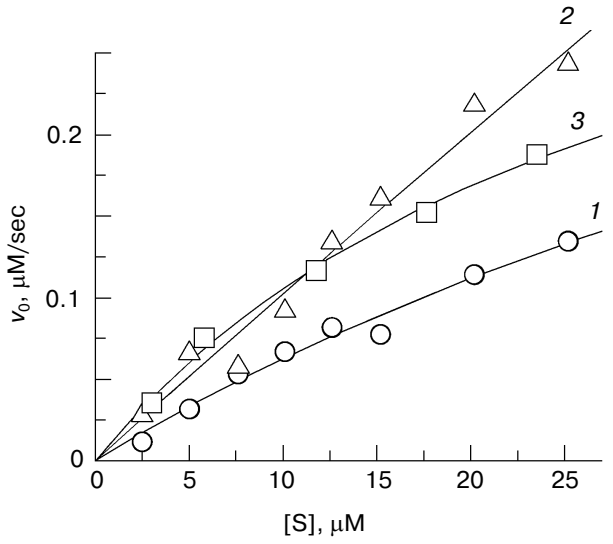

Fig. 1. Concentration dependence of the initial rate of peroxidase-catalyzed oxidation of 1-naphthol (*1*), 2-naphthol (*2*), and 4-hydroxybiphenyl (*3*). Conditions: 50 mM acetate buffer, pH 5.5, 25°C. PO (nM): 0.51 (*1*, *2*) and 1 (*3*). H_2O_2, 100 μM.

Peroxidase turnover number in 1- and 2-naphthol oxidation reactions. TN is the ratio between the amount of oxidized naphthol and the amount of inactivated enzyme (Eq. (7)). To determine TN, naphthol, hydrogen peroxide, and PO were incubated in the reactor for 15 min. Changes in enzyme activity and 1-naphthol concentration during the incubation and also the influence of albumin on these processes are presented in Fig. 3. Ratios of initial concentrations of peroxidase and naphthol are 4 nM : 50 μM and 10 nM : 100 μM during 1-naphthol oxidation; 4 nM : 50 μM and 5 nM : 100 μM during 2-naphthol oxidation. It was found that 1 molecule of PO during the oxidation of 1-naphthol and 2-naphthol makes 5500-7700 and 8800-10,000 turnovers, respectively. The introduction of 20 nM of albumin into the incubation mixture increases peroxidase turnover to 10,000 during 1-naphthol oxidation and to 11,000 during 2-naphthol oxidation. By the increasing the introduced albumin concentration to 200 nM, the TN increases to 28,000 or 39,000 for 1-naphthol and 2-naphthol, respectively, i.e., by 3-4 times.

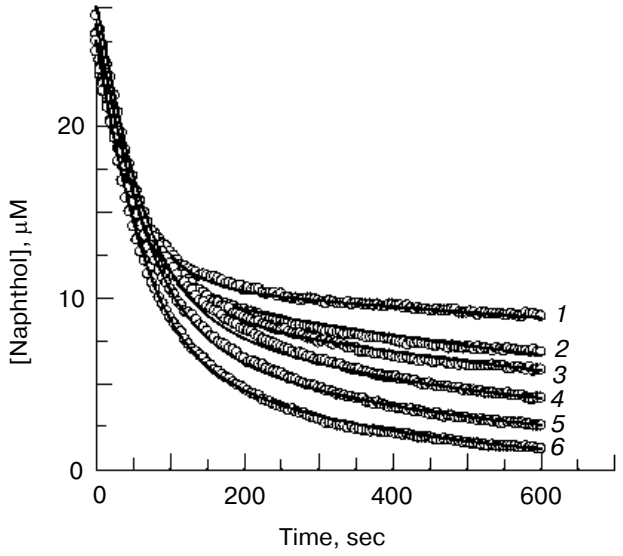

Fig. 3. Kinetics of 1-naphthol oxidation by peroxidase in the presence of HSA. Conditions: 50 mM acetate buffer, pH 5.5, 25°C. Concentrations: 25 μM naphthol, 1 nM PO, 100 μM H_2O_2. HSA (nM): 0 (*1*), 4 (*2*), 8 (*3*), 12 (*4*), 16 (*5*), 32 (*6*).

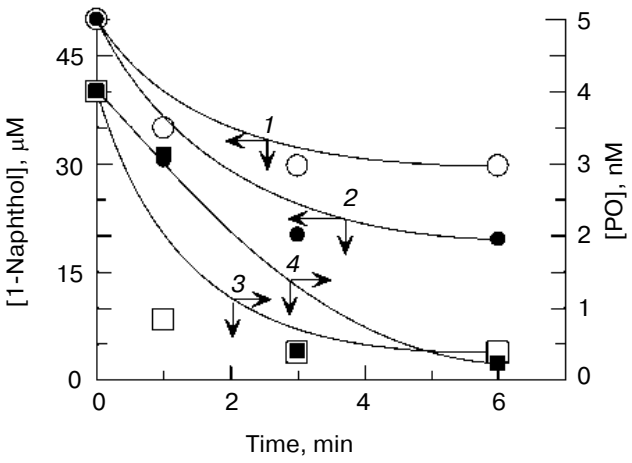

Fig. 4. Change in 1-naphthol (*1*, *2*) and active PO (*3*, *4*) concentration during incubation in the absence (*1*, *3*) and in the presence (*2*, *4*) of BSA. Conditions: PO, 4 nM (*1-4*); 1-naphthol, 50 μM (*1-4*); BSA, 20 nM (*2*, *4*); H_2O_2, 100 μM (*1-4*). Other conditions as in Fig. 1.

Influence of albumin on kinetics of naphthol oxidation by peroxidase. Naphthol oxidation curves in the presence of albumin in peroxidase oxidation reactions are presented in Fig. 4. The curves demonstrate that albumin has virtually no affect on the initial rate of the process; however, it increases the naphthol conversion degree.

The minimal kinetic scheme which could explain the obtained experimental data includes bi-molecular reaction of peroxidase oxidation with hydrogen peroxide, naphthol oxidation by oxidized form of peroxidase, peroxidase reaction with naphthol oxidation product, and product–product interaction resulting in the formation of a polymer (Eqs. (1)-(5)). The influence of albumin on the process is described by the following scheme:

$$\text{Product} + \text{albumin} \rightarrow [\text{product–albumin}], \quad (8)$$

$$[\text{Product–albumin}] + \text{product} \rightarrow \text{albumin} + \text{polymer}. \quad (9)$$

According to the scheme (1)-(5) and using Eq. (6), the kinetic parameters at different albumin concentrations (0-140 nM) were obtained. The analysis of these results reveals that the value of bimolecular constant k_{ox} is 5.5 ± 0.5 $\mu M^{-1} \cdot sec^{-1}$, which is the same for 1-naphthol and 2-naphthol. Unlike k_{ox}, values of k_{red} constant are different for 1-naphthol and 2-naphthol. Numerically, they are 13.6 ± 1.6 and 54.0 ± 1.7 $\mu M^{-1} \cdot sec^{-1}$ for 1-naphthol and 2-naphthol respectively, i.e., 4 times lower for 1-naphthol than for 2-naphthol. Values of k_{in} for 1-naphthol and 2-naphthol are $(1.1 \pm 0.3) \cdot 10^{-3}$ and $(0.86 \pm 0.24) \cdot 10^{-3}$ $\mu M^{-1} \cdot sec^{-1}$, respectively. The obtained k_{ox} constant is virtually the same as given in the literature [22, 23], which means that the suggested scheme indeed corresponds to the process of CI peroxidase complex formation. Difference in k_{red} and k_{in} values is connected to reactive ability of 1- and 2-naphthol. However, the mechanism of naphthol oxidation product interaction with peroxidase and influence of albumin on this interaction is still not clarified. It is possible that albumin participates as an activator for product recombination but is not consumed in this process [12, 13].

Kinetics of peroxidase-catalyzed naphthol oxidation in the presence of PEI and PL. The influence of PEI on naphthol oxidation by peroxidase is shown in Fig. 5, a and b. The initial rate of 1-naphthol oxidation was 0.07 μM/sec, and the value for 2-naphthol was 0.19 μM/sec. After 3 min, the concentration of 1-naphthol decreased from 25 to 18.6 μM, and 2-naphthol concentration decreased from 25 to 13.3 μM. It was found that PEI in the 0.075-5.0 nM concentration range decreases the initial reaction rate and oxidation degree of 1- and 2-naphthol. At PEI concentration higher than 0.02 μM, the initial reaction rate (Fig. 5a) and amount of oxidized naphthol (Fig. 5b) increase. The same influence was found for PL. Within PL concentration range of 0.47-1.55 μM, the initial rate of naphthol oxidation decreases 2.5 and 2 times for 1- and 2-naphthol, respectively. Increasing the PL concentration in the 3-7 μM range does not significantly increase the initial reaction rate. The kinetic characteristics of naphthol oxidation by peroxidase at different PEI and PL concentrations are presented in Table 1.

Influence of polymers on light scattering during peroxidase-catalyzed substrate oxidation. While studying

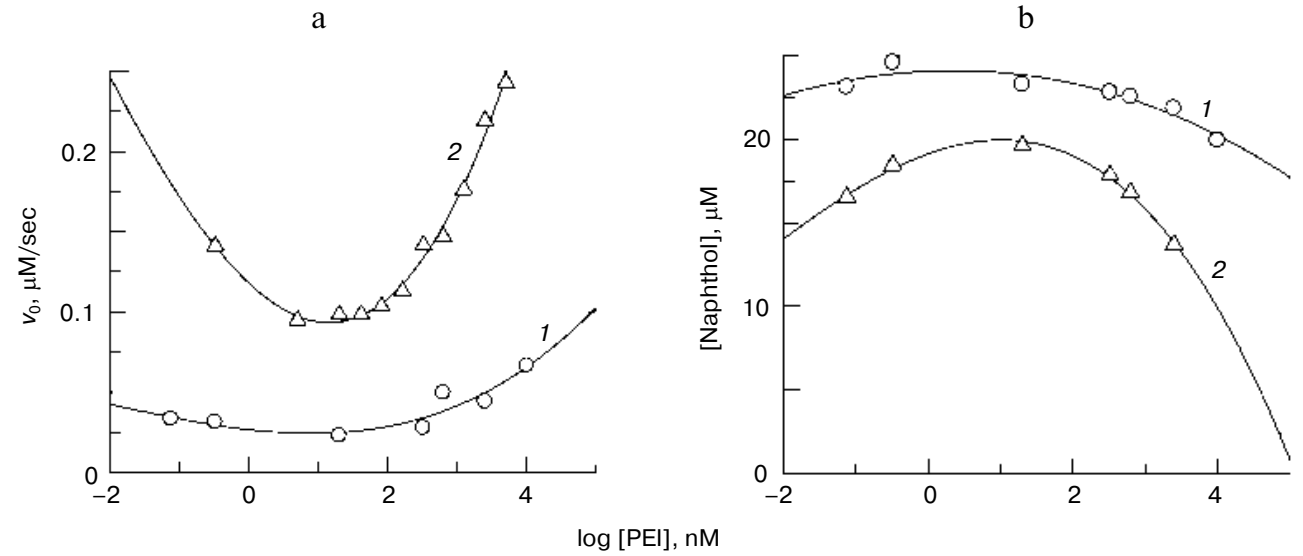

Fig. 5. Dependence of initial rate (a) and the amount of oxidized substrate (b) on PEI concentration in the reactions of 1-naphthol (*1*) and 2-naphthol (*2*) oxidation by peroxidase. Conditions the same as in Fig. 1.

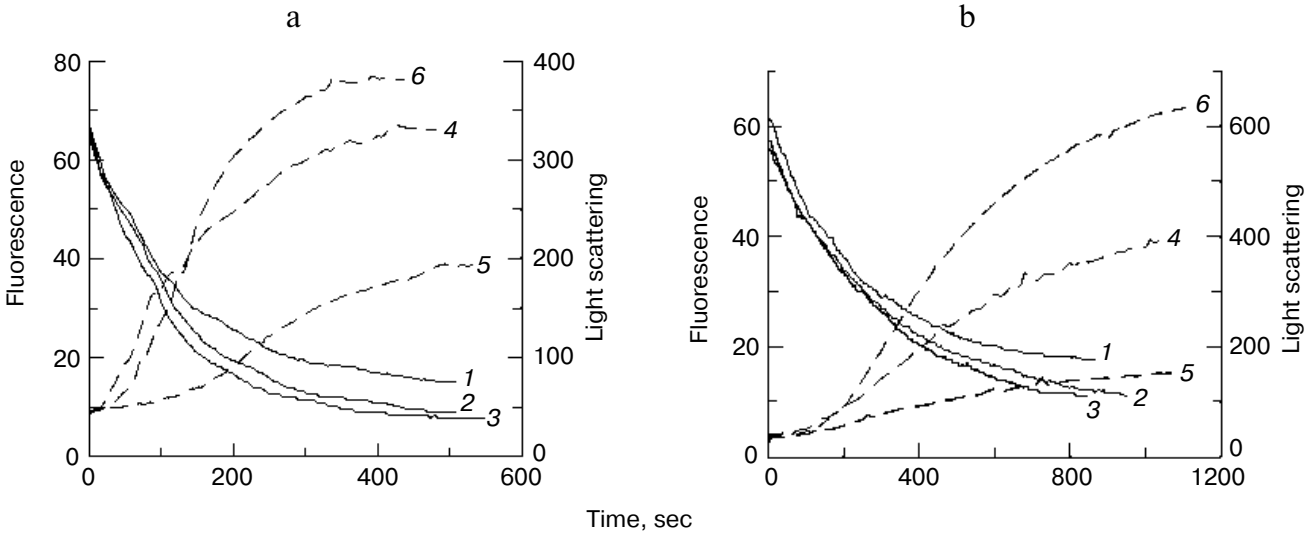

Fig. 6. Influence of BSA (*2*, *5*) and PEG (*3*, *6*) on fluorescence (*1-3*) (left ordinate) and light scattering intensity (*4-6*) (right ordinate) in the oxidation of 1-naphthol (a) and 2-naphthol (b) by peroxidase. Conditions: 25 μM naphthol, 200 nM BSA, 20 nM PEG, 1 nM PO, 100 μM H_2O_2, 50 mM acetate buffer, pH 5.5, 25°C.

substrate oxidation by peroxidase, an increase in light scattering intensity with crossed monochromators was observed. During the oxidation reaction of naphthols and 4-hydroxybiphenyl, the growth of light scattering intensity was proportional to the decrease in fluorescence intensity. Introduction of albumin or polyethylene glycol into the reaction mixture increased the amount of oxidized substrate and light scattering intensity. Light scattering intensity in the presence of BSA in the reaction mixture was 2 times lower than in the presence of PEG; however, both the initial reaction rate and the amount of oxidized naphthol in the presence of these two polymers were practically the same (Fig. 6, a and b).

The amount of microparticles formed was measured and calculated as well as their volume in buffer solutions of naphthol and 4-hydroxybiphenyl, reaction mixtures of naphthol and 4-hydroxybiphenyl, and reaction mixtures of naphthol and 4-hydroxybiphenyl containing PEG after the kinetic curves were completely saturated. The results of this investigation are presented in Table 2.

Table 1. Kinetic characteristics of peroxidase-catalyzed naphthol (25 μM) oxidation in the presence of PEI and PL (100 μM H_2O_2, 1 nM PO, 50 mM acetate buffer, pH 5.5, 25°C)

Substrate	Polymer	Concentration, μM	k_{ox}, $\mu M^{-1} \cdot sec^{-1}$	k_{red}, sec^{-1}	k_{in}, $\mu M^{-1} \cdot sec^{-1}$	k_p, $\mu M^{-1} \cdot sec^{-1}$
1-Naphthol	PEI	0	5.2	7.0	0.0038	—
»	»	$7.5 \cdot 10^{-5}$	5.2	7.0	0.056	—
»	»	$32.5 \cdot 10^{-5}$	5.2	5.0	0.08	—
»	»	0.02	5.2	7.5	0.075	—
»	»	0.16	5.2	7.8	0.21	0.97
»	»	0.325	5.2	7.8	0.1	0.19
»	»	0.625	5.2	7.8	0.1	0.13
»	»	2.5	5.2	6.0	0.06	0.035
»	»	10	5.2	10.5	0.072	0.02
»	PL	0	5.0	10.0	0.00195	—
»	»	0.47	5.0	10.0	0.08	0.2
»	»	1.55	5.0	11.0	0.002	0.0075
»	»	3.0	5.0	10.35	0.096	0.096
»	»	5.0	5.0	10.0	0.035	0.035
»	»	7.0	5.0	10.0	0.062	0.062
2-Naphthol	PEI	0	4.9	35.0	0.002	—
»	»	$7.5 \cdot 10^{-5}$	4.9	35.0	0.074	—
»	»	$32.5 \cdot 10^{-5}$	5.0	35.0	0.0075	—
»	»	0.02	5.0	34.0	0.011	—
»	»	0.16	5.5	48.0	0.0011	0.093
»	»	0.325	5.5	40.0	0.0082	0.02
»	»	0.625	5.6	41.0	0.0064	0.01
»	»	2.5	5.5	40.0	0.0034	0.0019
»	PL	0	5.0	23.0	0.0027	—
»	»	0.47	5.0	21.0	0.017	0.089
»	»	1.55	5.0	22.0	0.08	0.12
»	»	3.0	5.0	21.0	0.053	0.041
»	»	5.0	5.0	20.0	0.044	0.023
»	»	7.0	4.8	21.1	0.027	0.012

The introduction of PEG into the reaction mixture did not affect size-distribution of the particles, but their number. In the case of naphthol oxidation in the presence of PEG, the number of particles in the investigated solution decreases compared to the number of particles which are formed during naphthol oxidation in the absence of PEG, but the number of larger particles (31.6-100 μm) increases. Their volume compared to the theoretical data increases by two orders of magnitude. In the case of 4-hydroxybiphenyl, size distribution of particles formed in the presence of PEG does not change and varies in 3.1-31.6 μm range; the number of particles in 1 cm^3 of reaction mixture increases (Fig. 7).

DISCUSSION

Inactivation of peroxidase is observed both in the case of oxidation of phenols and different amines and in the case of oxidation of naphthols [24, 25]. Thorough investigation of peroxidase-catalyzed oxidation of naphthols and 4-hydroxybiphenyl revealed that the enzyme is inactivated during the oxidation reaction, which is common for enzymatic oxidation of phenolic substrates [26]. Insoluble polymers are the most often found oxidation products. To explain the mechanism of enzyme inactivation, enzymatic "self-destruction" was proposed. The decrease in enzyme activity during oxidation of phenols is

Table 2. Influence of PEG on the number and volume of microparticles formed during 1-naphthol (25 μM) and 4-hydroxybiphenyl (10 μM) oxidation by peroxidase (100 μM H_2O_2, 1 nM PO, 50 mM acetate buffer, pH 5.5, 25°C)

Substrate	Number of particles, N/cm^3	Volume of particles, cm^3
Acetate buffer	$2.5 \cdot 10^3$	—
1-Naphthol	$4.6 \cdot 10^3$	—
1-Naphthol/H_2O_2/PO	$1.5 \cdot 10^4$	$1.1 \cdot 10^{-4}$
1-Naphthol/H_2O_2/PO + PEG	$1.7 \cdot 10^4$	$6.1 \cdot 10^{-4}$
4-Hydroxybiphenyl	$4.4 \cdot 10^3$	—
4-Hydroxybiphenyl/H_2O_2/PO	$6.8 \cdot 10^3$	$1.8 \cdot 10^{-6}$
4-Hydroxybiphenyl/H_2O_2/PO + PEG	$11.2 \cdot 10^3$	$1.5 \cdot 10^{-5}$

associated with enzyme inactivation during irreversible reaction between the enzyme and an intermediate oxidation product (phenoxyl radical). A consequence of this interaction is the formation of a covalent bond between oxidized substrate (phenoxyl radical) and the enzyme [24, 25, 27]. On the basis of these results, it can be assumed that during peroxidation of naphthols the enzyme inactivation by naphthoyl radical (which is formed via one-electron oxidation of naphthol) occurs. It acts as an agent "exterminating" the enzyme activity.

From studies of naphthol oxidation by laccase, it is known that addition of proteins and water-soluble polymers such albumin, polyethylene glycol, ficoll, etc. into the reaction mixture decreases the enzyme inactivation and allows increasing substrate conversion degree [16]. The influence of albumin and certain water-soluble polymers (PEI, PL) on the kinetics of naphthol oxidation by peroxidase was investigated. One of the results obtained was that albumin efficiently inhibited peroxidase inactivation (Fig. 3) and increased the conversion of naphthol (Fig. 4).

Under stationary conditions, the rate of recombination may depend on product–albumin complex formation (Eq. (8)), which is supposedly formed via hydrophobic interactions, where albumin does not participate in subsequent processes. Kinetic analysis carried out in this work allows quantitative characterization of the process through which a reactively active product (naphthoyl radical [28]) binds to albumin, which perhaps is a main reason for the inhibition of peroxidase inactivation. Addition of polymers such as PEI or PL into the reaction mixture decreased the initial rate of naphthol oxidation by laccase [16, 28]. The influence of PEI on the oxidation of naphthols by peroxidase was ambiguous. A low concentration of introduced PEI (lower than 5 nM) decreased the initial rate and degree of naphthol conversion, while increasing PEI concentration increased these parameters. This influence can be associated with the presence of positively charged groups on PEI. Since an isoelectric point of PO is located in the 3.5-3.8 range, PEI molecules are electrostatically attracted to PO, which is carrying negative charge, and probably block it. The mechanism of this process is not completely clear and requires further investigation.

However, there is another hypothesis about the mechanism of enzyme inactivation. According to it, microparticles (microaggregates) are formed during the oxidation reaction, which is proved by the increased intensity of light scattering. Enzyme molecules are adsorbed on the surface of particles, which have a certain charge. Around a microparticle (as any heterogeneous surface) a stagnant (Nernstian) layer is formed, through which a substrate transfer is subjected to diffusion limitations [29].

The results obtained along with the literature data demonstrate that different factors affect the oxidation of substrates by peroxidase and number and size of microparticles formed. These are buffer concentration, pH and ionic strength of the solution [30], nature of the substrates and introduced catalytically inactive polymers, their molecular weight [31], etc.

A dependence of substrate conversion degree on acetate buffer concentration was observed during the study of the kinetics of substrate oxidation by peroxidase. The investigation of amount of oxidized 1-naphthol and

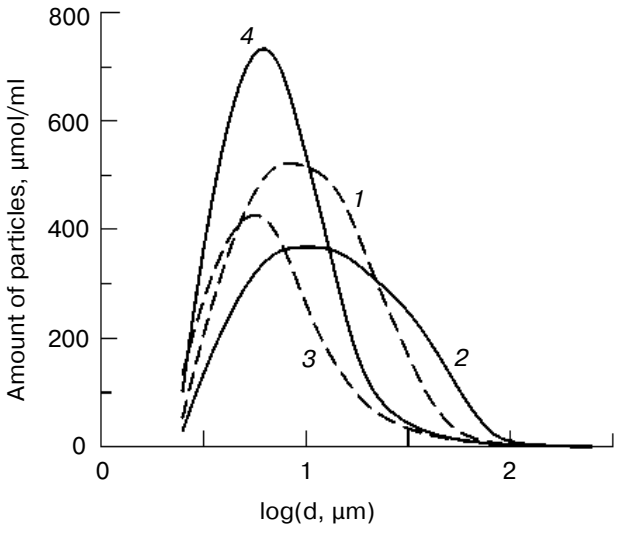

Fig. 7. Particle distribution by concentration in a volume unit and by size in peroxidase-catalyzed oxidation of 1-naphthol (*1*), 1-naphthol in the presence of 10 μM PEG (*2*), 4-hydroxybiphenyl (*3*), and 4-hydroxybiphenyl in the presence of 10 μM PEG (*4*). Conditions: 25 μM 1-naphthol, 10 μM 4-hydroxybiphenyl, 10 μM PEG, 100 μM H_2O_2, 1 nM PO, 50 mM acetate buffer, pH 5.5, 25°C.

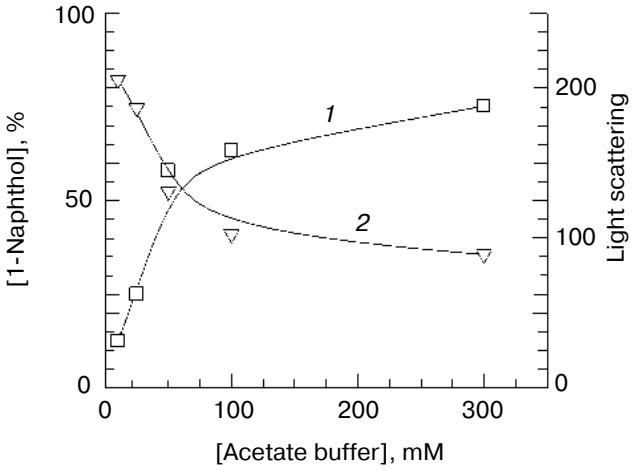

Fig. 8. Influence of acetate buffer concentration on the conversion degree (*1*) and light scattering intensity (*2*) in the reaction of 1-naphthol oxidation 500 sec after the reaction was started. Conditions the same as in Fig. 6.

dependence of light scattering intensity on the concentration of acetate buffer in the 10-300 mM range revealed that conversion degree of 1-naphthol and intensity of light scattering decrease with increasing buffer concentration (Fig. 8). The concentration of acetate buffer also affects 4-hydroxybiphenyl oxidation. Increasing buffer concentration decreases the amount of oxidized 4-hydroxybiphenyl, but with increase in the light scattering intensity.

A detailed analysis of data from the calculation of particle number and size in the investigated solutions was performed. It was revealed that the influence of PEG is not the same for naphthols and 4-hydroxybiphenyl. Addition of PEG into the reaction mixture of 4-hydroxybiphenyl oxidation caused twofold increase in particle number. On the other hand, the addition of PEG into the naphthol oxidation medium leads to a decrease in particle number and increase in their size-distribution interval. It can be assumed that during the peroxidase-catalyzed substrate oxidation polymers are formed, which are further aggregated into microaggregates by means of hydrophobic interactions. However, the microaggregates are not compact—they probably have a "loose" structure that gives an "increased" particle size. PEG or other globular polymers, which are introduced into the solution, become nucleation centers for the polymers formed during the reaction. Perhaps they prevent the adsorption of enzyme molecules on the microaggregates. In the absence of such polymers, the nucleation centers are apparently the enzyme molecules.

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