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Inhibition of Peroxidase-Catalyzed Oxidation of 3,3′,5,5′-Tetramethylbenzidine by Aminophenols

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Abstract—Peroxidase-catalyzed oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) was inhibited by *o*-aminophenol (AP), 2-amino-4-*tert*-butylphenol (ATBP), 2-amino-4,6-di-*tert*-butylphenol (ADTBP), and 4-*tert*-butylpyrocatechol (TBP). Inhibitors were characterized by inhibition constant K_i and stoichiometric coefficient *f*, the number of radicals terminated by one inhibitor molecule. The most efficient inhibitor is ADTBP characterized by K_i = 36 μM in 0.015 M phosphate citrate buffer, pH 6.0, at 20°C. According to their antiradical efficiency, the studied inhibitors can be arranged as follows: ADTBP > ATBP > AP > TBP. The role of the NH_2 group in the inhibitory capacity of aminophenols is discussed. Using gas–liquid chromatography, kinetics of consumption of the initial components and accumulation of the reaction products on peroxidase-catalyzed oxidation of the TMB–TBP pair was studied; the data clarify the stages of a complex process of co-oxidation of amines and phenols.

Key words: horseradish peroxidase, tetramethylbenzidine, aminophenols, inhibition, inhibition constants, stoichiometric inhibition coefficients

Peroxidase-catalyzed amine oxidation in the presence of phenols is a fundamental problem of peroxidase catalysis [1, 2]. This problem is also of practical importance because amine–phenol pairs are used in enzyme immunoassay of many antigens [3] and in general antioxidant activity tests of human and animal biological liquids, food, wines, juices, and natural biopreparations in which an amine is a substrate and phenol is a "calibrating" inhibitor (its antiradical activity is compared with the inhibitory properties of the analyzed biological liquids) ([4, 5] and references cited therein). We have systematically studied the kinetics of peroxidase-catalyzed co-oxidation of amine–phenol pairs for more than ten years [1-3, 5-14].

Oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB), one of the most widely used horseradish peroxidase (HP) substrates, was efficiently inhibited by gallic acid [6] and its polydisulfide [7, 8], 2-amino-4-nitrophenol (ANP) and its polydisulfide [9, 10], propyl gallate [11], 1-amino-2-naphthol-4-sulfonic acid and its polydisulfide [12], 2,4-dinitrosoresorcinol and polydisulfides of resorcinol and of 2,4-dinitrosoresorcinol [13]. We also studied alkyl-substituted pyrocatechols and hydroquinone as inhibitors of peroxidase-catalyzed TMB oxidation [2, 14]. To quantitatively characterize the efficiency of phenolic inhibitors in peroxidase-catalyzed oxidation of TMB and other HP substrates, we used the inhibition constant (K_i) and stoichiometric inhibition coefficient (*f*), the number of radicals terminated by one inhibitor molecule [1, 2, 5-14]. In all cases, polydisulfide phenol derivatives were more efficient than analogs of their monomeric units and had lower K_i and higher *f* values. Resorcinol polydisulfide (K_i = 0.78 μM, *f* = 76 [13]) and gallic acid polydisulfide (K_i = 1.33 μM, *f* = 37.6 [7, 8]) appeared to be the most efficient inhibitors; they can be used for complete termination of peroxidase-catalyzed TMB oxidation on multi-

Abbreviations: AP) *o*-aminophenol; ADTBP) 2-amino-4,6-di-*tert*-butylphenol; ATBP) 2-amino-4-*tert*-butylphenol; ANP) 2-amino-4-nitrophenol; HP) horseradish peroxidase; TBP) 4-*tert*-butylpyrocatechol; TBBQ) 4-*tert*-butyl-1,2-benzoquinone; TMB) 3,3′,5,5′-tetramethylbenzidine; PCB) phosphate citrate buffer; *f*) stoichiometric inhibition coefficient; K_i) inhibition constant, μM; GLC) gas-liquid chromatography.

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ple automated identification of many antigens by enzyme immunoassay.

In all TMB–phenol pairs studied by us earlier [1, 2, 5-14], the hydroxy groups of the substituted phenols and polyphenols are the radical-accepting centers: there are two of them for alkyl substituted pyrocatechols [2, 14] and also 2,4-dinitrosoresorcinol [13], four for gallic acid [6] and propyl gallate [11], and many more for polydisulfides of substituted phenols and polyphenols [7-10, 12, 13]. Study of peroxidase-catalyzed co-oxidation of TMB–aminophenol pairs with phenol component containing two different radical-accepting groups, OH and NH_2, is of great interest. That is why we chose *o*-aminophenol (AP), 2-amino-4-*tert*-butylphenol (ATBP), and 2-amino-4,6-di-*tert*-butylphenol (ADTBP) as potential inhibitors of peroxidase-catalyzed TMB oxidation and compared the inhibitory effect of ATBP on TMB oxidation with the effect of 4-*tert*-butylpyrocatechol (TBP) containing the hydroxy group instead of the amino group in ATBP.

In the present work, we studied the nature and parameters of inhibition of peroxidase-catalyzed TMB oxidation by *o*-aminophenol and its *tert*-butyl-substituted derivatives by kinetic methods and compared the inhibitory effects of ATBP and TBP on peroxidase-catalyzed TMB oxidation to choose the optimal calibrating inhibitors for general antioxidant activity tests of human and animal biological fluids.

MATERIALS AND METHODS

Reagents. In this work, we used horseradish peroxidase (HP) (EC 1.11.1.7) of A grade with optical purity indicator RZ 2.4 from Biolar (Latvia). Enzyme concentration was determined spectrophotometrically using the molar extinction coefficient $\varepsilon = 102{,}000\ M^{-1}\cdot cm^{-1}$ at the maximum of the Soret band (403 nm) [15]. We used diluted H_2O_2 as an oxidant; H_2O_2 concentration was determined spectrophotometrically using $\varepsilon_{230} = 72.1\ M^{-1}\cdot cm^{-1}$ [16]. Water-soluble TMB·2HCl from Sigma (USA) was used as the reducing HP substrate.

To prepare 0.015 M phosphate citrate buffer, pH 6.0 (PCB), we used the salts and citric acid from Reakhim (Russia). Dimethylsulfoxide, ethanol, and toluene from Reakhim were distilled before use.

Phenolic inhibitors. The UV spectrum of *o*-aminophenol (AP) of analytically pure grade from Reakhim with molecular mass 109 daltons had two extinction maxima at 244 and 284 nm ($\varepsilon_{284} = 2500\ M^{-1}\cdot cm^{-1}$) in 5% dimethylsulfoxide. 4-*tert*-Butylpyrocatechol (TBP) with molecular mass 166 daltons from Reakhim was additionally purified as described earlier [17]. 4-*tert*-Butyl-1,2-benzoquinone (TBBQ), a product of complete oxidation of TBP, as well as 2-amino-4-*tert*-butylphenol (ATBP) and 2-amino-4,6-di-*tert*-butylphenol (ADTBP), were synthesized as described earlier [18-20].

Peroxidase-catalyzed oxidation of TMB and TMB–AP, TMB–ATBP, TMB–ADTBP, and TMB–TBP pairs was monitored spectrophotometrically at 20°C using a Specol-211 spectrophotometer with thermostatted cuvette (Carl Zeiss, Germany).

Initial PCB, H_2O_2, HP, TMB, and TBP solutions were prepared in distilled water; AP, ATBP, and ADTBP solutions were prepared in ethanol.

On oxidation of TMB and TMB–AP pair, the reaction mixture with total volume 1 ml contained 0.019 M PCB (0.8 ml), 0.01 M TMB (0.05 ml), 0.02 M H_2O_2 (0.05 ml), 10 nM HP (0.02 ml), water (0.03 ml), and AP solution in ethanol (0.05 ml). Before addition of H_2O_2, the mixture was incubated for 3 min at 20°C. In a typical experiment on oxidation of TMB and TMB–AP pair the final concentrations of reagents in 0.015 M PCB, pH 6.0, were as follows: 0.2 nM HP, 1 mM H_2O_2, 0.5 mM TMB, 5% ethanol, and variable AP concentrations (30-90 μM).

On oxidation of TMB–ATBP, TMB–ADTBP, and TMB–TBP pairs, ATBP, ADTBP, and TBP concentrations were varied in the intervals 20-70, 10-50, and 20-80 μM, respectively; this was due to the limited solubility of the inhibitors.

Peroxidase-catalyzed TMB oxidation in the presence and in the absence of inhibitors was performed for 1-2 min; the optical absorption of the product of TMB oxidation was recorded at its maximum, 655 nm (A_{655}). The rates v_0 were calculated using $\varepsilon_{655} = 39{,}000\ M^{-1}\cdot cm^{-1}$ [15].

It is very important that under the peroxidase-catalyzed oxidation conditions the initial inhibitors and the products of their conversion did not contribute to the optical absorption band of the product of TMB oxidation (655 nm) for 1-2 min, that is, a correct spectral monitoring of the reducing HP substrate during the enzymatic process was achieved.

Characterization of inhibition of peroxidase-catalyzed TMB oxidation. To determine the type of inhibition, we plotted the data in Lineweaver–Burk coordinates. To determine K_i, we used the Dixon method [21]. We realize that K_i values are arbitrary, but nonetheless they adequately account for the antiradical efficiency of aminophenols and many phenol inhibitors used by us [1, 2, 6-14].

To determine the *f* values, we used the theory of the inhibitory mechanism of free-radical processes developed by N. M. Emanuel and coworkers [22] and the relations:

$$\Delta\tau = f\cdot[InH]_0/v_i\ , \qquad (1)$$

$$v_0 \approx v_i = f\cdot(v_i/f)\ , \qquad (2)$$

where $\Delta\tau$ is the induction period in accumulation of the product of TMB oxidation and v_i is the rate of initiation of radicals approximately equal to the rate of TMB oxidation in the absence of inhibitor. We have described the details of the method earlier [1, 2, 6-14].

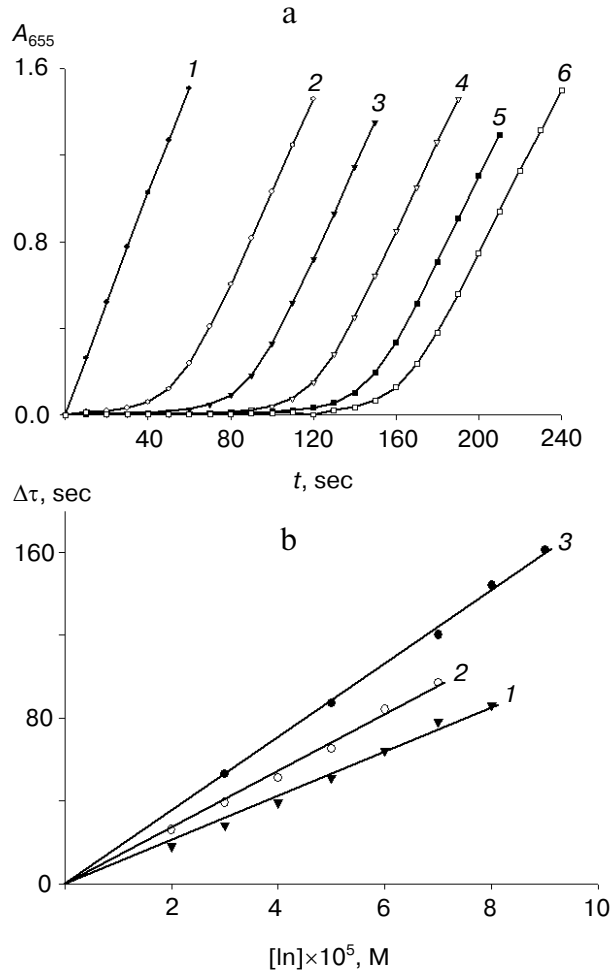

Fig. 1. a) Optical absorption A_{655} showing growth of the product of TMB (0.5 mM) oxidation at 20°C. AP concentrations (μM): *1*) 0; *2*) 30; *3*) 50; *4*) 70; *5*) 80; *6*) 90. The reaction mixture contained 0.015 M PCB, pH 6.0, 5% ethanol, 0.2 nM HP, and 1 mM H_2O_2. b) The induction period Δτ at 20°C versus the initial concentrations of TBP (*1*), ATBP (*2*), and AP (*3*). The reaction mixture contained 0.015 M PCB, pH 6.0, 0.2 nM HP, 1 mM H_2O_2, and 0.5 mM TMB.

Study of peroxidase-catalyzed oxidation of TMB, TBP, and TMB–TBP pair by gas–liquid chromatography (GLC). TBP and TMB consumed during peroxidase-catalyzed oxidation and the oxidation product, 4-*tert*-butyl-1,2-benzoquinone (TBBQ), were quantitatively analyzed using a Shimadzu GC-17A chromatograph (Japan) with flame ionization detector. Formation of the product of TMB oxidation was monitored spectrophotometrically as described in [15]. Preliminary experiments on GLC separation and quantitative analysis of TBP, TMB, and TBBQ demonstrated the following. Complete chromatographic separation and correct GLC analysis of TBP and TBBQ is attained using a DB-5 column from J and W Scientific (USA) 30 m in length and 0.54 mm in diameter (F = 1.5 μm). The flow rate of nitrogen used as carrier gas was 3.1 ml/min, ejection 10 : 1; T_{inject} = 270°C, T_{detect} = 250°C; T_{col}: 100→250°C (8°C/min).

TMB consumption was quantitatively analyzed on an RTX-1 column from RESTEK (USA) 30 m in length and 0.32 mm in diameter (F = 0.5 μm). The flow rate of nitrogen used as carrier gas was 1.0 ml/min, ejection 23 : 1; T_{inject} = 290°C, T_{detect} = 280°C; T_{col}: 100→300°C (6°C/min, for 5 min).

To prepare the calibrating solutions, TBP, TBBQ, and TMB samples were dissolved in toluene. For consumed TBP and TMB and formed TBBQ, we obtained strictly linear calibration plots "peak height–concentration".

Individual TBP, TMB, and TMB–TBP mixtures were oxidized at 17 or 20°C in 0.015 M PCB, pH 6.0, usually at the following reagent concentrations: 7 nM HP, 10 mM H_2O_2, 4.0 mM TBP, and 0.5 mM TMB. The reaction was terminated by addition of 200 μl of 2 M H_2SO_4 to 1 ml of the reaction mixture after 30, 60, 120, 150, 180, 210, 240, 300, and 600 sec. Then 87 mg of $NaHCO_3$ was added into the test tubes, and the reaction mixture was extracted with 1 ml of toluene. The toluene extracts were analyzed by GLC using various columns as described above.

RESULTS AND DISCUSSION

Inhibition of peroxidase-catalyzed oxidation of tetramethylbenzidine by aminophenols. Kinetic curves of A_{655} growth on peroxidase-catalyzed oxidation of TMB–AP pair at increasing AP concentrations from 30 to 90 μM are presented in Fig. 1a. A linear dependence of the induction period Δτ on AP concentration is observed

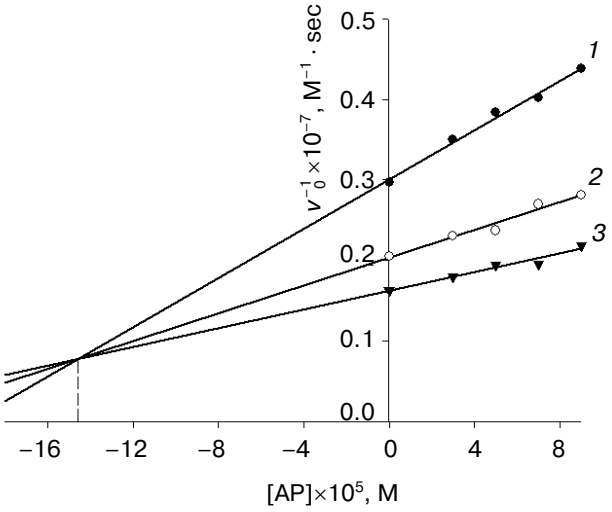

Fig. 2. Reciprocal rate of peroxidase-catalyzed TMB oxidation versus AP concentration at 20°C. TMB concentrations (mM): *1*) 0.15; *2*) 0.28; *3*) 0.50. The reaction mixture contained 0.015 M PCB, pH 6.0, 5% ethanol, 0.2 nM HP, and 1.0 mM H_2O_2.

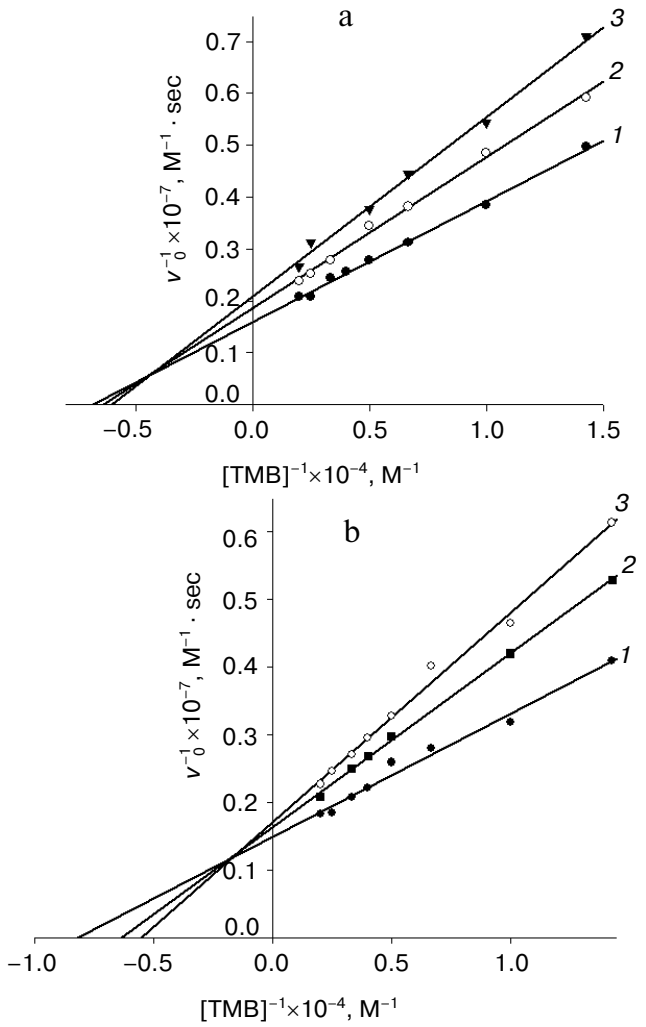

Fig. 3. Reciprocal rate of peroxidase-catalyzed TMB oxidation versus its reciprocal concentration at 20°C in the presence of 0, 20, and 70 μM (*1-3*, respectively) ATBP (a) and 0, 27, and 45 μM (*1-3*, respectively) ADTBP (b). The reaction mixture contained 0.015 M PCB, pH 6.0, 5% ethanol, 0.2 nM HP, and 1.0 mM H_2O_2.

(Fig. 1b), which corresponds with the theory of the inhibitory mechanism [22]. Using this figure and Eqs. (1) and (2), we obtained that $f = 2.3$, that is, one AP molecule reacts on average with 2-3 cation-radicals $(TMB)^{\bullet+}$ via the exchange mechanism:

$$(TMB)^{\bullet+} + AP \rightarrow TMB + (AP)^{\bullet}. \qquad (3)$$

The rates of TMB oxidation versus its concentration in Lineweaver–Burk coordinates in the absence and in the presence of AP at various concentrations prove the mixed type of inhibition of TMB oxidation by *o*-aminophenol [23]. Dependencies of v_0^{-1} on AP concentration (in Dixon coordinates) are presented in Fig. 2. Using this figure, we obtained that $K_i = 146$ μM, that is, AP is an inhibitor of medium efficiency in the process of peroxidase-catalyzed TMB oxidation.

The data on oxidation of the TMB–ATBP pair in double reciprocal coordinates are presented in Fig. 3a. Using Fig. 1b and Eqs. (1) and (2), we obtained that $f = 1.6$ that is, one ATBP molecule on average reacts with 1-2 cation-radicals $(TMB)^{\bullet+}$. Using Fig. 4 we obtained that $K_i = 98$ μM.

According to an analogous scheme, we studied peroxidase-catalyzed oxidation of the TMB–ADTBP pair (Fig. 3b). As in the first two cases, the inhibition type of TMB oxidation appeared to be mixed. From kinetic data, we determined that $f = 1.4$ and $K_i = 36$ μM. Kinetic parameters of inhibition of peroxidase-catalyzed TMB oxidation by three aminophenols are presented in the table. It should be noted that change in conditions of peroxidase-catalyzed oxidation of TMB–AP pair results in K_i increase from 146 to 460 μM, that is, the inhibitory action of AP on peroxidase significantly decreases, whereas changes in the *f* values are negligible.

According to the table, the inhibitory effect of aminophenols increases in the series AP < ATBP < ADTBP, that is, insertion of one and two *tert*-butyl substituents into the aromatic ring significantly increases antiradical efficiency of aminophenols. The rate constants of reaction (3) increase in the same series, AP < ATBP < ADTBP. This result is not surprising because the *tert*-butyl substituent activates the HO- and NH_2-groups in their homolytic reactions with radicals [24]. That is why ADTBP is a rather efficient inhibitor of peroxidase-catalyzed TMB oxidation as compared with AP.

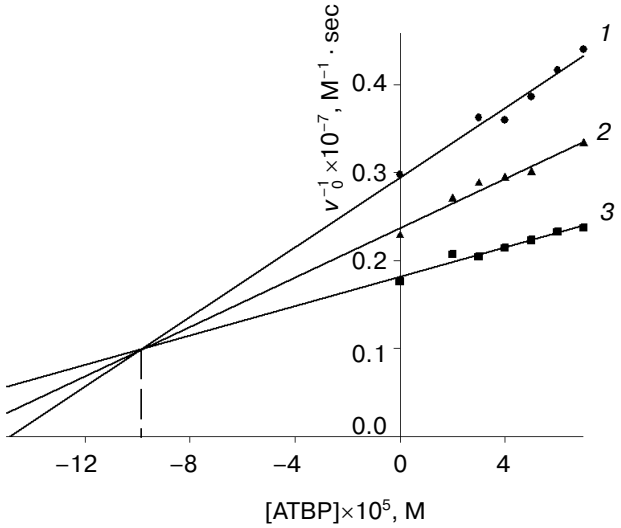

Fig. 4. Reciprocal rate of peroxidase-catalyzed TMB oxidation versus ATBP concentration at 20°C. TMB concentrations (mM): *1*) 0.15; *2*) 0.28; *3*) 0.50. The reaction mixture contained 0.015 M PCB, pH 6.0, 5% ethanol, 0.2 nM HP, and 1.0 mM H_2O_2.

Kinetic parameters of inhibition of peroxidase-catalyzed TMB oxidation by aminophenols and TBP at 20°C (the reaction mixture contained 0.015 M PCB, pH 6.0, 0.2 nM HP, 1 mM H_2O_2, 0.5 mM TMB, and 5% ethanol in case of AP, ATBP, and ADTBP)

Inhibitor	Inhibition type	K_i, μM	f
AP*	mixed	460	2.6
AP	mixed	146	2.3
ANP**	competitive	160	0.3
ATBP	mixed	98	1.6
ADTBP	mixed	36	1.4
TBP	noncompetitive	188	0.8

* AP was oxidized in 0.015 M PCB, pH 6.4, containing 5% dimethylformamide, 5% dimethylsulfoxide, 0.8 nM HP, 1 mM TMB, and 1 mM H_2O_2 [23].

** ANP was oxidized in 0.01 M phosphate buffer, pH 6.4, containing 10% dimethylformamide, 1 nM HP, 0.5 mM TMB, and 1 mM H_2O_2 [9].

The role of the NH_2 group in aminophenol molecules in the exchange reaction (3) between aminyl cation-radical $(TMB)^{\bullet+}$ and aminophenols is of particular interest. Does the NH_2 group enhance or attenuate the antiradical activity of aminophenols compared with the HO group in 4-*tert*-butylpyrocatechol (TBP)? To answer this question, we studied kinetics of peroxidase-catalyzed oxidation of the TMB–TBP pair under conditions analogous to those for oxidation of the TMB–aminophenol pairs.

Inhibition of peroxidase-catalyzed oxidation of tetramethylbenzidine by 4-*tert*-butylpyrocatechol (TBP). It is known that *tert*-butyl-substituted phenols are substrates for peroxidase [25, 26]. That is why TBP at increasing concentrations inhibited peroxidase-catalyzed TMB oxidation, which is characterized by an induction period in accumulation of the product of TMB oxidation. Duration of the induction period Δτ linearly depends on $[TBP]_0$ (Fig. 1b). Using this figure and Eqs. (1) and (2), we obtained that $f = 0.8$; this means that one TBP molecule on average reacts with one cation-radical $(TMB)^{\bullet+}$. The rate of TMB oxidation versus its initial concentration in the double reciprocal coordinates at various TMB concentrations is presented in Fig. 5a. These dependences prove noncompetitive character of inhibition of peroxidase-catalyzed TMB oxidation in the presence of TBP unlike the mixed type of inhibition of this process by three aminophenols. We presented the data in Dixon coordinates (Fig. 5b) and thus obtained that $K_i = 188$ μM, that is, the inhibitory effect of TBP is a half that of its analog ATBP (table). Thus, under analogous conditions the antiradical activity of ATBP, a *tert*-butyl-substituted aminophenol, is twice as large as that of 4-*tert*-butyl-substituted pyrocatechol. The f value for ATBP is twice as large as that for TBP.

So, the presence of the neighboring HO and NH_2 groups as the radical-accepting centers in ATBP molecule is preferred over two neighboring HO groups in the TBP molecule.

To elucidate the steps of a complex process of peroxidase-catalyzed co-oxidation of TMB and substituted phenols, it is necessary to study kinetics of consumption of TMB and phenol and accumulation of the reaction products in one experiment. As an example, it is worth-

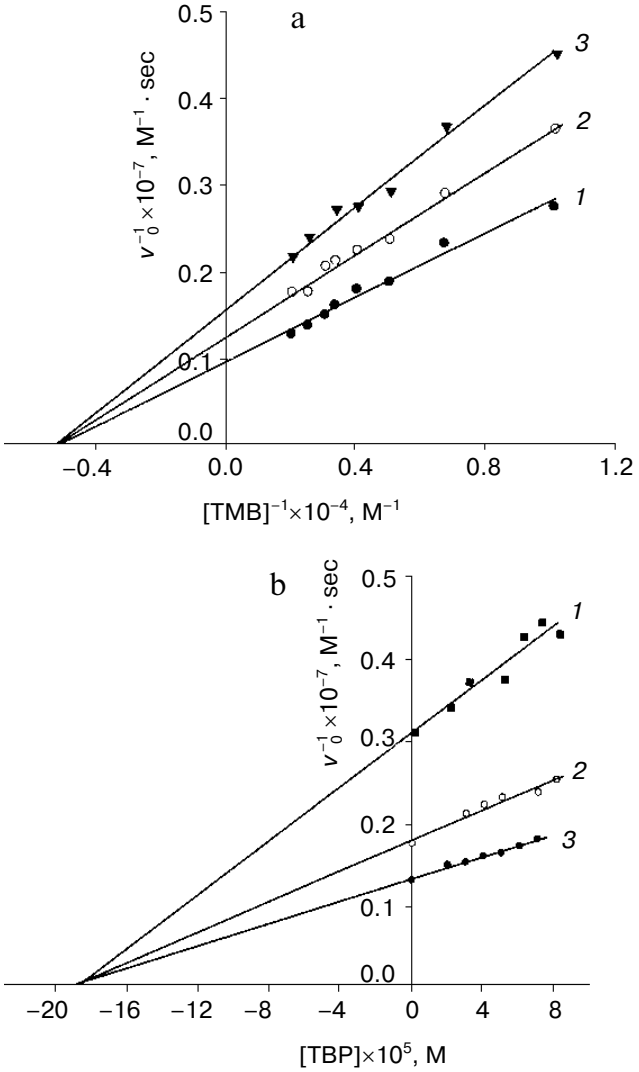

Fig. 5. Reciprocal rate of TMB oxidation versus its reciprocal concentration (a) and TBP concentration (b) in peroxidase-catalyzed oxidation of the TMB–TBP pair at 20°C. a) TBP concentrations (μM): *1*) 0; *2*) 30; *3*) 60. b) TMB concentrations (mM): *1*) 0.2; *2*) 0.3; *3*) 0.5. The reaction mixture also contained 0.015 M PCB, pH 6.0, 0.2 nM HP, and 1 mM H_2O_2.

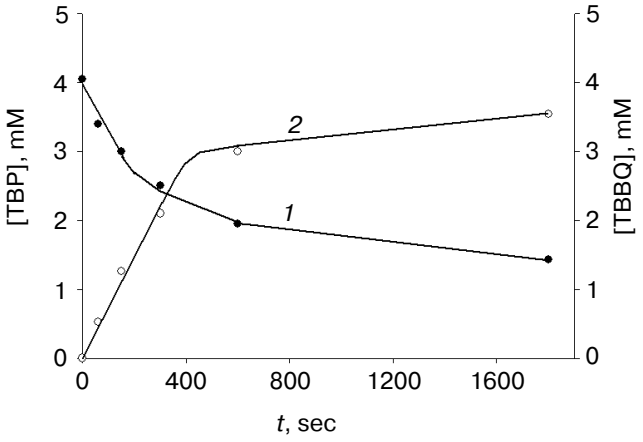

Fig. 6. Kinetics of TBP consumption (*1*) and TBBQ accumulation (*2*) in peroxidase-catalyzed TBP oxidation at 20°C. Concentrations: 0.015 M PCB, pH 6.0, 4 mM TBP, 7 nM HP, and 10 mM H_2O_2.

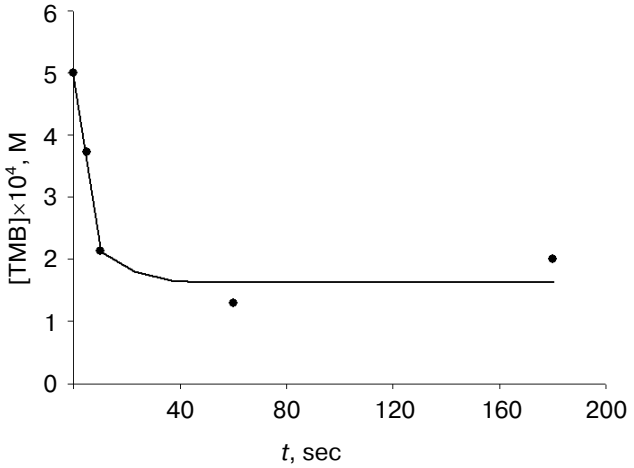

Fig. 7. Kinetics of TMB consumption in its peroxidase-catalyzed oxidation at 20°C. Concentrations: 0.015 M PCB, pH 6.0, 0.5 mM TMB, 7 nM HP, and 10 mM H_2O_2.

while to study peroxidase-catalyzed oxidation of the TMB–TBP pair, because the products of oxidation of TMB are well known and can be easily identified spectrophotometrically [27], and the final product of peroxidase-catalyzed TBP oxidation is a corresponding quinone (TBBQ), which can be identified chromatographically as well as TBP itself. Hence, we studied comparative kinetics of peroxidase-catalyzed oxidation of TMB, TBP, and TMB–TBP pair by gas–liquid chromatography (GLC).

Study of kinetics of peroxidase-catalyzed oxidation of TMB, TBP, and TMB–TBP pair by GLC. We obtained chromatograms of consumed TBP and the product of its oxidation TBBQ at 20°C (the reaction mixture contained 7 nM HP, 4 mM TBP, and 10 mM H_2O_2). TBP and TBBQ were completely separated. Kinetic curves of TBP consumption and TBBQ accumulation under the same conditions are presented in Fig. 6: the initial rate of TBP consumption is $(6.8 \pm 0.3) \cdot 10^{-6}$ M·sec^{-1} and the rate of TBBQ accumulation $(7.3 \pm 0.3) \cdot 10^{-6}$ M·sec^{-1}, that is, the rates of the substrate conversion and the product accumulation are very close. This means that TBBQ is the only final product of TBP oxidation under the reaction conditions.

We also obtained chromatograms of consumed TMB on its peroxidase-catalyzed oxidation at the following concentrations: 7 nM HP, 0.5 mM TMB, and 10 mM H_2O_2 (20°C). Chromatographic identification of the products of TMB oxidation appeared to be impossible using an RTX-1 column. However, an intermediate cation-radical $(TMB)^{\bullet+}$ and the final product of the reaction can be easily identified spectrophotometrically [27]. The initial rate of TMB consumption (Fig. 7) is rather high under the experimental conditions ($2.9 \cdot 10^{-5}$ M·sec^{-1}).

Kinetic dependences presented in Fig. 8 demonstrate that during 200 sec, TBP is consumed with the constant rate $1.7 \cdot 10^{-5}$ M·sec^{-1}, and TBBQ, the product of its oxidation, is formed with the constant rate $1.6 \cdot 10^{-5}$ M·sec^{-1}. Simultaneously up to 30 sec, TMB is consumed with the rate $5.3 \cdot 10^{-6}$ M·sec^{-1} and then during 30-200 sec an induction period is observed in its conversion, but after 200 sec when TBP is consumed almost completely, TMB oxidation continues with a rather lower rate ($2.2 \cdot 10^{-7}$ M·sec^{-1}). We did not identify the products of cross-recombination of phenoxyl radicals $(TBP)^{\bullet}$ and aminyl cation-radicals $(TMB)^{\bullet+}$ on chromatograms.

Thus, being monitored spectrophotometrically and chromatographically, dynamics of the process of peroxidase-catalyzed co-oxidation of TMB and TBP appears to be the same.

The reaction schemes and mechanisms of peroxidase-catalyzed co-oxidation of amines ($AmNH_2$) and phenols (PhOH) have been discussed many times in our previous works [1, 2, 6-14]. The pathway and characteristics of the process are mainly determined by non-enzymatic exchange reaction of the aminyl and phenoxyl radicals discovered by N. M. Emanuel and coworkers [28, 29]:

$$AmNH^{\bullet} + PhOH \leftrightarrow AmNH_2 + PhO^{\bullet}. \qquad (4)$$

If reaction (4) proceeds in the direct way, regeneration of amine and accelerated consumption of phenol is observed; this manifests itself in the induction period in formation of the products of amine oxidation. The induction period in formation of $(TMB)^{\bullet+}$ also appears on co-oxidation of TMB with four aminophenols and TBP, that is, the reactivity of this cation-radical is sufficient for oxi-

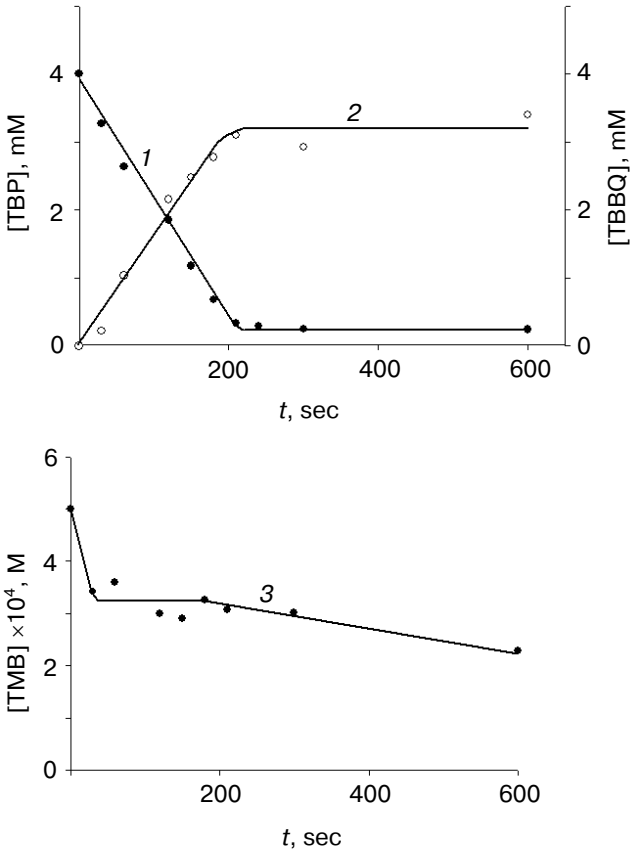

Fig. 8. Kinetics of consumption of TBP (*1*) and TMB (*3*) and accumulation of TBBQ (*2*) in peroxidase-catalyzed oxidation of the TMB–TBP pair at 17°C. Concentrations: 0.015 M PCB, pH 6.0, 0.5 mM TMB, 4 mM TBP, 7 nM HP, and 10 mM H_2O_2.

dation of all the above-mentioned phenols with various rates. The substituted phenols used in this reaction differ in their reactivity in relation to the radicals. The rate of reaction (4) depends on the conditions (pH, the presence of organic co-solvent) and on the structure of the phenolic inhibitor. The advantage of ATBP as an inhibitor of TMB oxidation over TBP can be caused by two reasons: first, that the effect of the neighboring NH_2 substituent on the reactivity of the HO group is higher than the effect of the neighboring hydroxy group; second, that the NH_2 group can become a reactive center in the case of aminophenols—this group can react with the radicals via the mechanism of electron transfer from the nitrogen atom with higher rate than the HO group reacting with the radicals via the mechanism of homolytic cleavage of the H–O bond.

As follows from the table, on peroxidase-catalyzed oxidation of TMP-substituted phenol pairs, various types of inhibition are observed: mixed for three aminophenols, noncompetitive for TBP, and competitive for 2-amino-4-nitrophenol (ANP). This means the following: TMB and TBP do not compete for binding to the active site of HP; this corresponds with data showing that amines and phenols have different binding sites in the hydrophobic channel of HP [30]; TMB and ANP compete for binding to the active site of HP; in the case of mixed inhibition of TMB oxidation by three aminophenols, a partial competition of the substrate for the binding sites of HP and competition of the substrate and inhibitors for the reactive intermediate forms of HP, compounds I and II, can take place. The mixed inhibition can be also caused by a loss in HP activity during the peroxidase-catalyzed process due to an interaction of the enzyme itself with the intermediate products of oxidation of aminophenols.

As follows from the table, aminophenol ADTBP is the most efficient inhibitor of peroxidase-catalyzed TMB oxidation (K_i = 36 μM). However, from a practical viewpoint, in general antioxidant activity tests of biological fluids, where TMB is a substrate and a substituted phenol is a calibrating inhibitor, ATBP and ANP are the most acceptable, because their inhibitory activities fall within an interval typical of such biological fluids as normal and pathological human serum [4, 5].

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REFERENCES

1. Metelitza, D. I., Karasyova, E. I., Grintsevich, E. E., and Thorneley, R. N. F. (2004) *J. Inorg. Biochem.*, **98**, 1-9.
2. Metelitza, D. I., Naumchik, I. V., Karasyova, E. I., Polozov, G. I., and Shadyro, O. I. (2003) *Prikl. Biokhim. Mikrobiol.*, **39**, 352-362.
3. Metelitza, D. I., Litvinchuk, A. V., and Savenkova, M. I. (1992) *Biokhimiya*, **57**, 103-113.
4. Metelitza, D. I., Eryomin, A. N., Sviridov, D. O., and Kamyshnikov, V. S. (2001) *Biochemistry (Moscow)*, **66**, 505-514.
5. Rus', O. B., and Metelitza, D. I. (2001) *Vestsi Akad. Navuk Belarusi. Ser. Khim. Navuk*, **4**, 75-82.
6. Karasyova, E. I., Nikiforova, T. V., and Metelitza, D. I. (2001) *Prikl. Biokhim. Mikrobiol.*, **37**, 472-479.
7. Karasyova, E. I., Losev, Yu. P., and Metelitza, D. I. (1997) *Biochemistry (Moscow)*, **62**, 1074-1081.
8. Karasyova, E. I., and Metelitza, D. I. (1999) *Biochemistry (Moscow)*, **64**, 54-60.
9. Karasyova, E. I., Nikiforova, T. V., Losev, Yu. P., and Metelitza, D. I. (1999) *Bioorg. Khim.*, **25**, 665-672.
10. Karasyova, E. I., Losev, Yu. P., and Metelitza, D. I. (2001) *Biochemistry (Moscow)*, **66**, 608-617.
11. Grintsevich, E. E., Senchuk, V. V., Puchkaev, A. V., and Metelitza, D. I. (2000) *Biochemistry (Moscow)*, **65**, 924-932.
12. Karasyova, E. I., Losev, Yu. P., and Metelitza, D. I. (2001) *Prikl. Biokhim. Mikrobiol.*, **37**, 713-721.
13. Karasyova, E. I., Losev, Yu. P., and Metelitza, D. I. (2002) *Bioorg. Khim.*, **28**, 147-155.
14. Naumchik, I. V., Karasyova, E. I., Metelitza, D. I., Polozov, G. I., and Shadyro, O. I. (2004) *Bioorg. Khim.*, **30**, 537-546.
15. Metelitza, D. I., Savenkova, M. I., and Kurchenko, V. P. (1987) *Prikl. Biokhim. Mikrobiol.*, **23**, 116-124.

16. Nikol'skii, B. P. (ed.) (1967) *Chemist's Handbook* [in Russian], Vol. 4, Khimiya, Leningrad, p. 919.
17. Belostotskaya, I. S., and Komissarova, N. L. (1971) *Izv. Akad. Nauk SSSR*, *Ser. Khim.*, **12**, 2816-2817.
18. Magnusson, R. (1960) *Acta Chem. Scand.*, **14**, 1643.
19. Albert, H. E. (1954) *J. Am. Chem. Soc.*, **76**, 4985-4988.
20. Vol'eva, B. P., Prokof'eva, T. I., Prokof'ev, A. I., Belostotskaya, I. S., Komissarova, N. L., and Ershov, V. V. (1995) *Izv. Akad. Nauk SSSR*, *Ser. Khim.*, **9**, 1789-1793.
21. Keleti, T. (1990) *Basics of Enzyme Kinetics* [Russian translation], Mir, Moscow, pp. 183-203.
22. Emanuel, N. M., and Buchachenko, A. L. (1982) *Chemical Physics of Aging and Stabilization of Polymers* [in Russian], Nauka, Moscow, pp. 239-308.
23. Naumchik, I. V., Karasyova, E. I., Metelitza, D. I., Polozov, G. I., and Shadyro, O. I. (2002) *Vestsi Akad. Navuk Belarusi. Ser. Khim. Navuk*, **3**, 85-91.
24. Ershov, V. V., Nikiforov, G. A., and Volod'kin, A. A. (1972) *Sterically-Hindered Phenols* [in Russian], Khimiya, Moscow.
25. Valoti, M., Sipe, H. J., Sgragli, G., and Mason, R. P. (1989) *Arch. Biochem. Biophys.*, **269**, 423-432.
26. Rodriques-Lopez, J. N., Gilabert, M. A., Tudela, J., Thorneley, R. N. F., and Garcia-Canovas, F. (2000) *Biochemistry*, **39**, 13201-13209.
27. Josephy, P. D., Eling, T., and Mason, R. P. (1982) *J. Biol. Chem.*, **257**, 3669-3675.
28. Karpukhina, G. V., Maizus, Z. K., and Emanuel, N. M. (1963) *Dokl. Akad. Nauk SSSR*, **152**, 110-114.
29. Karpukhina, G. V., Maizus, Z. K., and Emanuel, N. M. (1965) *Dokl. Akad. Nauk SSSR*, **160**, 158-162.
30. Kim, B. B. (1992) *Advances in Science and Technology. Biotechnology* [in Russian], Vol. 36, VINITI, Moscow, pp. 126-146.