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Peroxidase-catalyzed co-oxidation of 3,3',5,5'-tetramethylbenzidine in the presence of substituted phenols and their polydisulfides.

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Abstract

The steady-state kinetics of the horseradish peroxidase (HRP)-catalyzed oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) has been studied in the presence of 2-amino-4-nitrophenol (ANP), gallic acid (GA) or 4,4/-dihydroxydiphenylsulfone (DDS) and their polydisulfides poly(ADSNP), poly(DSGA), poly(DSDDS) at 20 °C in 10 mM phosphate buffer, pH 6.4, supplemented with 5-10% dimethylformamide. The second-order rate constants for the reactions of ANP, GA, poly (DSGA) and poly (DSDDS) with HRP-Compound I (κ_2) and Compound II (κ_3) have been determined at 25 °C in 10 mM phosphate buffer, pH 6.0 by stopped-flow spectrophotometry. ANP, GA and their polydisulfides strongly inhibited HRP-catalyzed TMB oxidation. Inhibition constants (K_i) and stoichiometric coefficients of inhibition (f) have been determined for these reactions. The most effective inhibitor was poly (DSGA) (K_i = 1.3 μM, f = 35.6). The oxidation of substrate pairs by HRP, i.e., TMB-DDS and TMB-poly (DSDDS) at pH 7.2 resulted in a ~8- and ~ 12-fold stimulation of TMB oxidation rates, respectively. The mechanisms of the HRP-catalyzed co-oxidation of TMB-phenol pairs are discussed.

Keywords: Horseradish peroxidase; Tetramethylbenzidine; Substituted phenols; Phenol's polydisulfides; Co-oxidation

1. Introduction

The peroxidase-catalyzed oxidation of amines in the presence of phenols is a basic scientific problem. This process is of commercial interest since such reactions form the basis of widely used quantitative photometric assays of phenols and naphthols [1] and enzyme-immunoassays (Elsa) of many protein antigens [2,3, and references therein].

We have previously shown that peroxidase-catalyzed co-oxidation of amines and phenols involves sequential and parallel enzymatic and non-enzymatic reactions [1-13]. The peroxidase-catalyzed oxidation of substrate pairs (i.e., 4-amino-antipyrine-phenols [1,2] and lumi-nol-phenols [2,3]) is characterized by an enhanced rate of amine oxidation. The co-oxidations of 3,3',5,5'-tetramethylbenzidine (TMB) with gallic acid (GA) [9], its polydisulflde poly(DSGA) [4,5,7], 2-amino-4-nitrophe-nol (ANP), and its polydisulflde (poly(ADSNP) [6,10], l-amino-2-naphthol-4-sulfonic acid and its polydisulfide [11], 2,4-dinitrosoresorcinol and polydisulflde derivatives of resorcinol and 2,4-dinitrosoresorcinol [12] and propylgallate [8] are characterized by a pronounced inhibition of the rate of oxidation of the amine component. The degree of inhibition of TMB oxidation depends on the phenol type and is much higher for polydisulfides inhibitors compared to the corresponding monomeric forms [4-12].

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Our observation that peroxidase-catalyzed co-oxidations of TMB with 4,4'dihydroxy-diphenylsulfone (DDS), its polydisulflde (poly(DSDDS)) [10] and poly(salicylic acid 5-aminodisulfide) [13] occurs with an enhanced rate of TMB oxidation provided evidence for the participation of the non-enzymatic radical exchange reaction shown in Eq. (1) [14,15].

$$PhO^{\cdot} + AmNH_2 \Longleftrightarrow PhOH + AmNH^{\cdot} \qquad (1)$$

The effect that a particular phenol will have on the peroxidase-catalyzed oxidation of a particular amine depends on the relative rates of the forward and back reactions of Eq. (1): if the reaction proceeds from left to right, amine oxidation will be accelerated and the phenol is regenerated (condition 1); conversely, if the reaction goes from right to left, phenol oxidation will be accelerated and the amine is regenerated (condition 2).

Clearly, to predict the mutual effect of phenols and amines during peroxidase-catalyzed co-oxidation, it is necessary to know the rate constants of the exchange reactions in both directions for each "amine-phenol" pair. However, these rate constants have often not been determined. Therefore, the selection of either "promoters" or "inhibitors" of peroxidase-catalyzed oxidation of aromatic amines is invariably empirical not least because the kinetics of co-oxidation are complex and depend on factors other than the exchange reaction shown in Eq. (1).

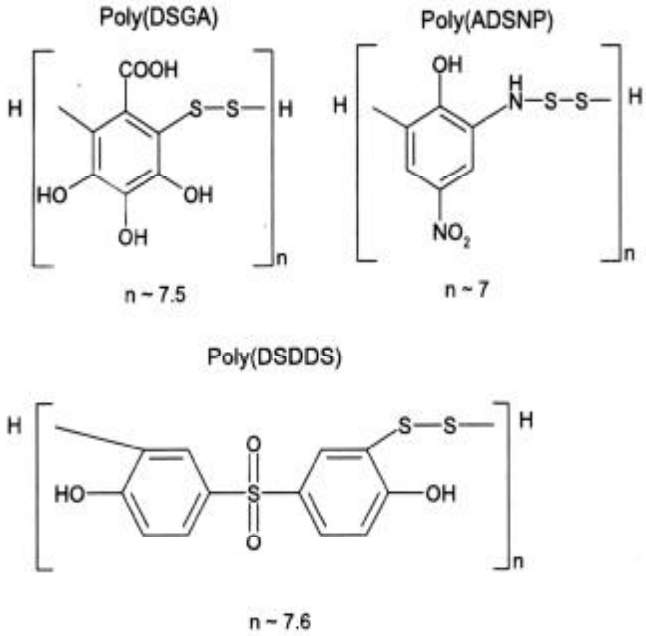

Fig. 1. Structures of phenol polydisulfides used in the peroxidase-catalyzed reactions.

The present work is a comparative study of the steady-state kinetics of the peroxidase-catalyzed co-oxidation of a single amine (TMB) with a range of phenols (ANP, GA, DDS) and their polydisulfides (poly-(ADSNP), poly(DSGA), poly(DSDDS)). In addition, the kinetics of oxidation of ANP, GA, poly(DSGA) and poly(DSDDS) by the intermediate forms of HRP, Compounds I *(E_1)* and II *{E_2)*, have been studied by stopped-flow spectrophotometry. These phenols and their polydisulfides were chosen because of their significant structural differences including a variable number of HO-groups (see Fig. 1) in order to provide mechanistic insights into the structural determinants of the HRP- catalyzed co-oxidation of "aromatic amine-substituted phenol" pairs.

The choice was also influenced by the high antioxi-dative efficiency of polydisulfides of substituted phenols and their successful use as bioantioxidants of lipid per-oxidation in cranio-cerebral traumas and by phenol's ability to diminish free radical formation in biochemical systems [4-12].

2. Materials and methods

2.1. Reagents

Horseradish peroxidase (EC 1.11.1.7, isoenzyme C) was purchased from Biozyme Ltd. (Blaenavon, Gwent, UK, Rz = 3.25) and Biolar (Olaine, Latvia, Rz = 2.75**).** The concentration of HRP was determined spectro-photometrically using the molar absorption coefficient at the Soret band maximum (403 nm) equal to 102,000 M^{-1} cm^{-1} [16].

Hydrogen peroxide was purchased from Aristar (UK) or Reakhim (Russia). The concentration of H_2O_2 was determined spectrophotometrically using r(203 nm) = 72.1 M^{-1} cm^{-1} [17].

TMB from Serva (Germany) was used as the reducing substrate of HRP. 2-Amino-4-nitrophenol (ANP), gallic acid (GA) and 4,4′dihydroxydiphenylsulfone (DDS) were purchased from Reakhim (Russia) and were of analytical grade purity. Dimethylformamide (DMF) from Reakhim was redis-

tilled before use. All the salts and bases used for the preparation of buffer solutions were of analytical grade from Reakhim (Russia).

2.2. Polydisulfides of substituted phenols

ANP polydisulfide (poly(ADSNP)) with an average molecular weight ~1400 Da (~7 monomer units) was prepared as described earlier [18]. Its UV-spectrum was characterized by four maxima in distilled water - 220, 255, 312 and 364 nm. The concentration of poly-(ADSNP) in water was determined using the molar extinction coefficient e_{312nm} = 35,000 M^{-1} cm^{-1}.

GA polydisulfide (poly(DSGA)) with an average molecular weight ~1760 Da (~7.5 monomer units) was synthesized as described previously [19]. The UV-spectrum of poly(DSGA) in water is characterized by two absorbance maxima with e_{312nm} = 295,107 M^{-1} cm^{-1} and e_{261nm} = 82,937 M^{-1} cm^{-1}.

DDS polydisulfide (poly(DSDDS)) with an average molecular weight ~2350 Da (7-8 monomer units) was synthesized as described earlier [20]. Stock solutions of poly(DSDDS) were prepared in DMF or ethanol.

UV-Vis spectrophotometry was carried out using a "Specord UV-VIS" instrument (Germany).

Steady-state kinetics of TMB oxidation in the presence and absence of phenols was studied at 20 °C in 10 mM phosphate buffer (pH 6.4) supplemented with 5% DMF and unless otherwise stated 1 nM HRP, 1 mM H_2O_2 and 0.5 or 1.0 mM TMB. The reaction was initiated by the addition of H_2O_2 and followed spectrophotometrically by recording the increase in optical density of the TMB oxidation product at the maximum of its absorption at 655 nm (A_{655}). It should be noted that the absorption bands of all the substituted phenols used and their oxidation products did not overlap with the 655 nm absorption band of the TMB oxidation product. The initial rate of TMB oxidation was calculated using the molar absorption coefficient of its product, 39,000 M^{-1} cm^{-1} [16]. The reactions were carried out in a thermostated cuvette of a Specol-211 device (Carl Zeiss, Germany). The initial rates were the mean of duplicate determinations.

2.3. Determination of the inhibitory parameters of phenols and their polydisulfides.

Inhibition constants K_i were determined from Dixon plots (v_0^{-1} vs. [InH]o) using the intercept of straight lines obtained for different TMB concentrations [21].

HRP-Compounds I and II are one electron oxidizing agents and generate free radicals as reaction products. Therefore, the Emanuel et al. [22,23] theory of inhibition is applicable to HRP catalyzed reactions, i.e., at a constant radical initiation rate v_i and with linear chain breaking, Eq. (2) applies

$$[\mathrm{InH}] = [\mathrm{InH}]_o - (v_i/f)\,t. \qquad (2)$$

Thus, the inhibitor is consumed with a constant rate V_i/f, where f is stoichiometric coefficient, i.e., the average number of free radicals terminated by one inhibitor molecule. The induction (lag) period A_T resulting from the inhibition of substrate oxidation is given by the following equation:

$$\Delta\tau = f[\mathrm{InH}]_o/v_i. \qquad (3)$$

This enables V_i/f, the free radical initiation rate, to be calculated to the same degree of accuracy as the value of f, which we have determined for TMB peroxidation.

For a steady-state free-radical reaction, the initial rate of substrate consumption is essentially equal to the free radical initiation rate Eq. (4) [22,23]

$$v_0 \approx (v_i/f)f. \qquad (4)$$

If v_0 is determined for TMB oxidation in the absence of the inhibitor InH and v_i/f is calculated from the lag phases observed with different InH concentrations using Eq. (3), then the stoichiometric coefficient f, a characteristic of a given inhibitor in a given experimental set, can readily be obtained using Eq. (4).

Note. In a case of TMB peroxidation the experimentally determined v_0 must be doubled, since the oxidation product, monitored spectrophotometrically (A_{655}), is formed from two cation-radicals $(TMB)^{\bullet +}$ [24].

2.4. Pre-steady-state kinetics.

Transient kinetics were monitored with a stopped-flow spectrophotometer (Model SF-61 DX2, Hi-Tech Scientific, UK). The light path was 1 cm and the sample temperature was 25 °C. All solutions were prepared using deionized water from a Milli-Q System (Milli-pore). Stock solutions of DDS and poly(DSDDS) were prepared in 10 mM phosphate buffer, pH 6.0, supplemented with 20% ethanol because of the low solubility of these compounds in water: the final ethanol concentration for all samples was 5%. In a typical experiment HRP (3.2-24.4 μM) was premixed with stoichiometric amount of H_2O_2 in the aging loop for 100-1000 ms and then was allowed to react with varying concentrations of substituted phenols in 10 mM phosphate buffer, pH 6.0.

The reduction of Compound I to Compound II was monitored at 411 nm (the isosbestic point between Compound II and the ferric HRP *(E))*, under our experimental conditions Compound I was stable for minutes. Compound II reduction to E was monitored at 430 nm.

The pseudo-first-order rate constants were calculated using an exponential curve-fitting program (kinetAsyst 3.0,Hi-Tech Scientific, UK). At least three determinations of pseudo-first-order rate constants (k_{obs}) were performed for each phenol concentration and the mean value was used in the calculation of the second-order rate constants k_2 and k_3 for Compounds I and II, which were determined from a slope of the line defined by a plot of k_{obs} vs. phenol concentration.

3. Results

3.1. Inhibition of TMB oxidation with ANP and poly(ADSNP)

The solubility of TMB and poly(ADSNP) in buffer solution is low: therefore, we used 1 mM phosphate buffer, pH 6.4 (PB) containing 10% DMF as a reaction medium. HRP catalyzed oxidation of TMB, in the presence of the inhibitor ANP, exhibited an induction period (AT) for product formation. The duration of the lag phase AT is linearly dependent on the ANP concentration (Fig. 2(a)) as is predicted by free radical kinetic theory [22,23]. The v_o value for the TMB oxidation in the absence of ANP, and the data of Fig. 2(a), give a factor f = 0.32 for ANP inhibition of TMB oxidation, i.e., only every third radical of the substrate reacts with an ANP molecule. To characterize the type of inhibition, we studied the initial rate of the TMB oxidation as a function of TMB concentration at increasing concentrations of ANP: Linewever-Burke plots of the data showed that ANP acts as a competitive inhibitor with respect to TMB (Fig. 2(b)) with K_i = 160 μM, i.e., ANP has a relatively low efficiency as an inhibitor of peroxidase catalyzed TMB oxidation.

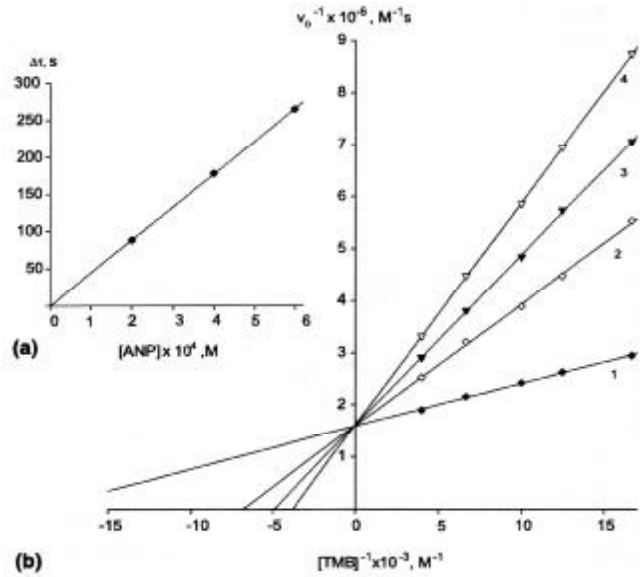

Fig. 2. (a) Concentration dependence of the induction period Δτ on $[ANP]_o$: [TMB] 0.06 mM. (b) Initial rate of TMB oxidation vs. substrate concentration in double reciprocal coordinates: 0.01 M phosphate buffer, pH 6.4, containing 10% DMF; [HRP] 1 nM, and $[H_2O_2]$ 1 mM, [ANP] mM: 0 (1), 0.2 (2), 0.4 (3) and 0.6 (4).

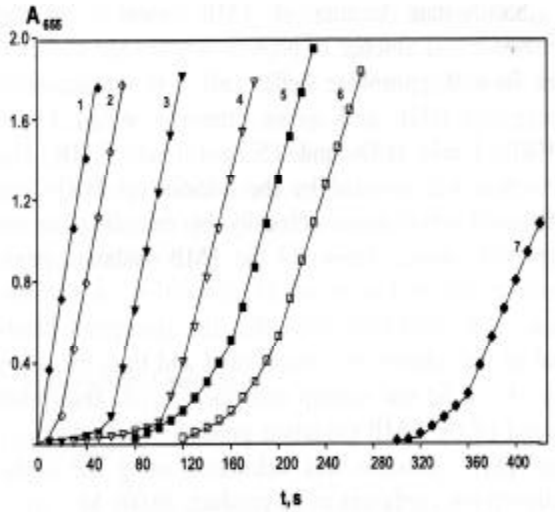

Fig. 3. Inhibition of TMB oxidation at increased GA concentrations. Kinetic curves of increase in absorbance of TMB oxidation product in 10 mM of PB, pH 6.4, [HRP] 1 nM, $[TMB]_o$, $[H_2O_2]_o$ 1 mM in the absence of GA (1) and in the presence of increasing GA concentrations: 10 (2), 30 (3), 60 (4), 80 (5), 100 (6) and 200 μM (7).

The rate of TMB oxidation is greatly decreased by poly(ADSNP) with pronounced lag phases dependent on the inhibitor concentration (data not shown). Using the analysis procedure described above we calculate f = 2.68, that is, 2-3 radicals of the substrate react with one molecule of the polymeric inhibitor in the peroxi-dase-catalyzed reaction. Linewever-Burke plots for TMB oxidation in the presence of varying concentrations of poly(ADSNP) show competitive inhibition with respect to TMB with K_i = 18 μM. Thus the efficiency of the poly(ADSNP) as an inhibitor is an order of magnitude higher than that of its monomeric analogue ANP (k_i = 160 μM).

3.2. Inhibition of TMB oxidation with GA and poly (DSGA)

Fig. 3 shows the kinetics of TMB oxidation in the absence (curve 1) and presence of GA concentrations in the range 10-200 μM (curves 2-7). The duration of the induction periods increased with increasing GA concentrations. The analysis procedure described above gave value of f = 2.6. Dixon plots (Fig. 4) gave competitive inhibition with K_i = 13.3 μM.

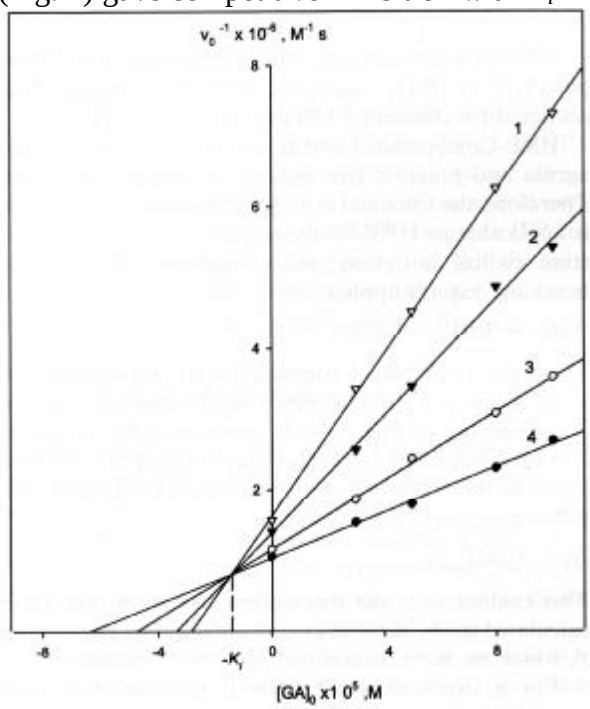

Fig. 4. Dixon plots for the dependence of the initial rates of TMB oxidation on initial $[GA]_0$ at increasing TMB concentrations: 0.2 (1), 0.3 (2), 0.5 (3) and 0.6 mM (4); 10 mM PB, pH 6.4, [HRP] 1 nM, $[H_2O_2]$ 1 mM.

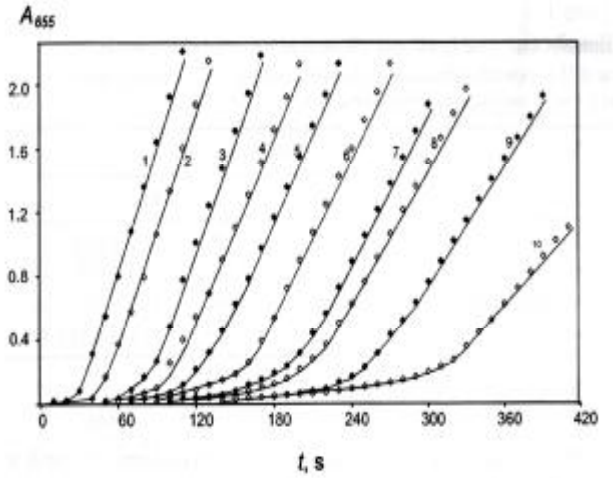

Fig. 5. Influence of increasing poly(DSGA) concentrations (μM) on the increase of absorbance of product of TMB (1 mM) oxidation in 10 mM PB, pH 6.4 ([HRP] 1 nM, $[H_2O_2]_0$ 1 mM): 1.42 (1), 2.27 (2), 3.84 (3), 4.25 (4), 5.68 (5), 7.0 (6), 8.52 (7), 9.93 (8), 11.36 (9) and 14.2 μM (10).

Fig. 5 shows the effect of replacing GA by poly(DSGA) (1.42-14.2 μM) at initial TMB and H_2O_2 concentrations of 1 mM. The linear dependence of the induction time (Δt) on the initial poly(DSGA) concentration gives V_i/f = 4.6 x 10^{-8} M s $^{-1}$. Under identical conditions in the absence of poly(DSGA) the initial rate of TMB oxidation was v_0 = 8.2 x 10^{-7} M s^{-1}. According to Eq. (4), f = (2 x 8.2 x 10^{-7})/(4.6 x 10^{-8}) = 35.6. This high value of f reflects the high inhibitory efficiency of poly (DSGA). For many inhibitors the value of f is about 2 [25]. Dixon plots (Fig. 6) show that poly(DSGA) is a competitive inhibitor of TMB oxidation with K_i = 1.3 μM which together with the high value of f = 35.6, makes poly(DSGA) a very potent inhibitor of TMB oxidation.

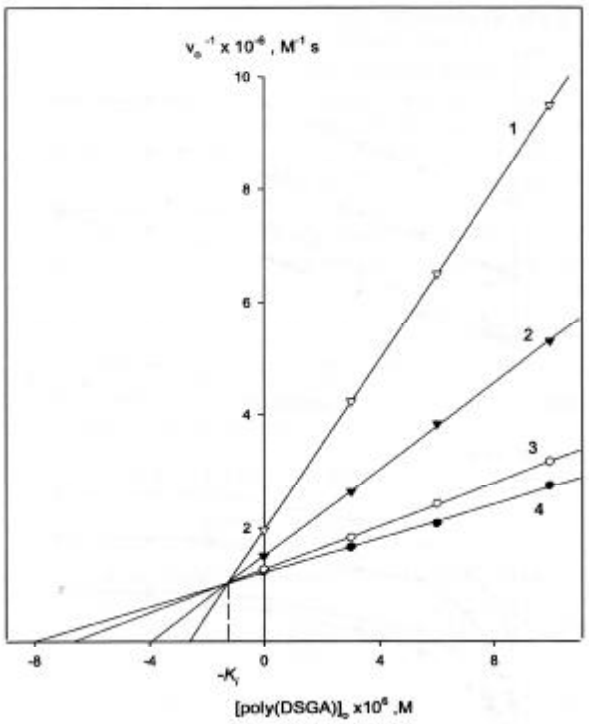

Fig. 6. Dixon plots of the inhibition of TMB oxidation by poly(DSGA) at increasing concentrations of TMB: 0.1 (1), 0.2 (2), 0.4 (3) and 0.5 (4) mM; [HRP] 1 nM, [H_2O_2] 1 mM.

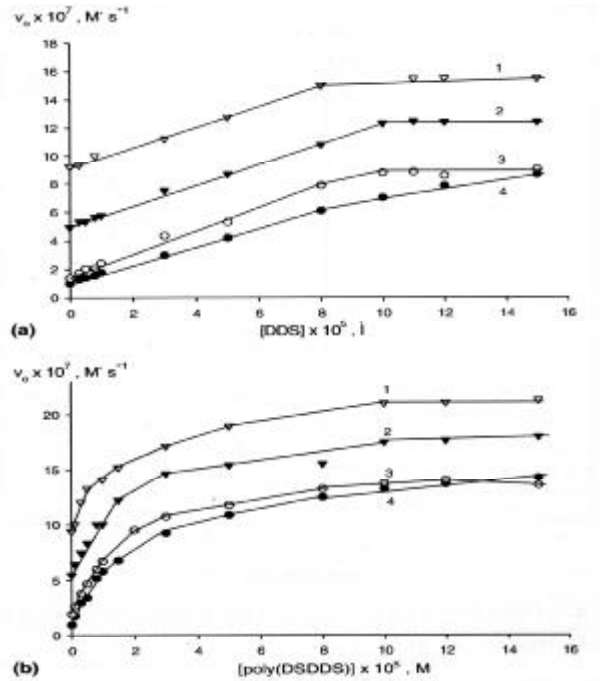

Fig. 7. Dependence of the initial rates of the HRP-catalyzed TMB oxidation in 10 mM PB, pH 6.4 with 5% DMF on concentrations of DDS (a) and poly(DSDDS) (b) at different pH values: 5.9 (1), 6.4 (2), 7.0 (3), and 7.2 (4); [HRP] 1 nM, [TMB]$_o$ 1 mM and [H_2O_2]$_o$ 1 mM.

3.3. Peroxidase-catalyzed co-oxidation of TMB with DDS and poly (DSDDS)

In 10 mM phosphate buffer (pH 6.4) supplemented with 5% DMF the initial rate of the HRP-catalyzed oxidation of TMB (1 mM) by hydrogen peroxide is directly proportional to the HRP concentration within the range of 0.1-1.5 nM. Under these conditions, DDS at concentrations of 50 and 100 μM increased the rate of TMB oxidation ~2- and 2.4-fold, respectively, i.e., this substituted diphenol activates the HRP-catalyzed TMB oxidation. Fig. 7(a) presents the dependences of the initial rate of TMB oxidation on the increasing concentration of DDS at different pH values within the range of 5.9-7.2. In all cases, the initial rate of TMB oxidation increased up to a limiting value at DDS concentrations >100 μM. For the co-oxidation of the TMB-DDS pair, the initial rate of TMB conversion significantly decreased with increasing pH from 5.9 to 7.2 at different concentrations of the diphenol component (DDS).

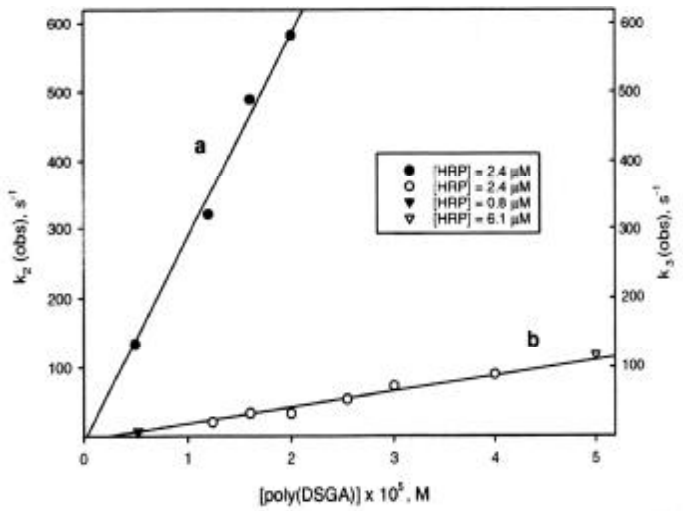

Fig. 8. Concentration dependence of the observed first-order rate constants for the reactions of Compound I (k_2(obs)) (line a, left-hand ordinate) and Compound II (k_3(obs)) (line b, right-hand ordinate) with poly(DSGA) (5–50 μM): 25 °C, 10 mM PB, pH 6.0 [H_2O_2]$_o$ = 2.4 μM; [HRP] as indicated on the figure.

Note. The HRP-catalyzed oxidation of the TMB alone exhibits a similarly dependence on pH. Data for similar experiments with poly (DSDDS) are presented in Fig. 7(b). In all cases, the polymer phenol activates the oxidation of TMB, with limiting values attained at poly (DSDDS) concentrations >100 μM. Values of v_o decrease with increasing pH at different concentrations of poly(DSDDS). At pH 7.2, the HRP-catalyzed co-oxidation of TMB-DDS and TMB-poly(DSDDS) pairs is characterized by enhanced rates of TMB oxidation i.e., -8-fold for DDS and -12-fold for poly (DSDDS).

3.4. Pre-steady-state kinetics of reactions of the HRP-Compounds I and II with ANP, GA, poly (DSGA) and poly (DSDDS)

At 25 °C in 10 mM phosphate buffer (pH 6.0) the reaction of HRP-Compounds I and II with a range of phenolic substrates were studied by stopped-flow spectrophotometry. Fig. 8 shows the dependence of the pseudo-first-order rate constants for Compound I (k_2(obs)) and Compound II (k_3(obs)) reduction, respectively, on the poly(DSGA) concentration from which the bimolecular rate constants given in Table 1 were calculated. Analogous data for the reactions of GA with the corresponding bimolecular rate constants are given in Table 1 together with those calculated from experiments with ANP and poly (DSDDS).

Table 1
Bimolecular rate constants of interaction of HRP Compounds I (k_2) and II (k_3) with substituted phenols and polydisulfides of phenols: 25°, 0.01 M phosphate buffer, pH 6.0

Phenols	Rate constants ($M^{-1} s^{-1}$)	
	k_2	k_3
GA	$(2.9 \pm 0.2) \times 10^6$	$(1.5 \pm 0.2) \times 10^5$
Poly(DSGA)	$(3.0 \pm 0.2) \times 10^7$	$(2.4 \pm 0.2) \times 10^6$
Poly(DSDDS)	$(2.0 \pm 0.2) \times 10^5$	$(3.1 \pm 0.2) \times 10^3$
ANP	–	$(1.1 \pm 0.2) \times 10^8$

We could not determine the rate constants k_2 and k_3 for poly(ADSNP) because this polydisulfide itself and the products of its peroxidation have strong absorption bands in the range 400-450 nm. The reaction of ANP with Compound I was complete within the stopped-flow instrument dead time and therefore we could only determine the value for the reaction with Compound II (k_3). We attribute the high value of k_3 = 1.1 x 10^8 M^{-1} s^{-1} to the presence of the 4-nitro-group in ANP. The reactions of poly(DSDDS) with HRP-Compound I and Compound II were carried out in 10 mM phosphate buffer (pH 6.0) supplemented by 5% ethanol because of the low solubility of this polydisulfide in aqueous media. The values of k_2 and k_3 for poly(DSGA) are 10- and 16-fold higher, respectively, than those for GA. The values of k_2 and k_3 for poly(DSGA) are 150- and 775-fold higher, respectively, than those for poly(DSDDS).

4. Discussion

Based on the results presented above and our previous work [1-13], we conclude that the HRP-catalyzed oxidation of TMB can be either inhibited or activated by the addition of phenolic compounds. The structure of the phenol determines whether inhibition or activation occurs. The data show that these effects are induced at concentrations of the phenol that are relatively low compared to that of the amine (TMB) undergoing oxidation. The kinetic analysis of such systems requires a consideration of radical initiation and termination processes and importantly in the context of this study, the exchange reaction between phenoxyl radical and the amine.

To explain the data presented in Table 2, it is necessary to consider the simplest scheme for the HRP-catalyzed co-oxidation of an "amine-phenol" pairs developed and discussed previously by ourselves [1-13] and others [26,27]. In Scheme 1, the following abbreviations are used: *E*, HRP; E_1 and E_2, active Compounds I and II of HRP; P_1 and P_2, oxidation products of phenols and amines, respectively; P_3 product of phenol and amine co-oxidation. Scheme 1 is divided into enzymatic and non-enzymatic reactions. Progress in protein engineering and the determination of the tertiary structures of peroxidases during recent years [28-30, and references therein] has resulted in a detailed elucidation of the molecular mechanism HRP and related peroxidases. T he phenols and their polydisulfides used in this study (Table 2) are unlikely to affect reaction (1), the rapid formation of Compound I by heterolytic cleavage of bound peroxide which occurs with k_1 = 2 x 10^7 M^{-1} s^{-1}) [26,27,30,31].

Table 2
The inhibition parameters of TMB oxidation by substituted phenols and their polydisulfides at 20 °C in 10 mM phosphate buffer, pH 6.4

Phenols	Number of monomer units (n)	f	K_i (μM)
ANP	1	0.32	160
Poly(ADSNP)	~7	2.68	18
GA	1	2.60	13.3
Poly(DSGA)	~7.5	35.6	1.3
DDS	1	~8-fold[a]	Activation
Poly(DSDDS)	~7.6	~12-fold[a]	Activation

[a] Data obtained at pH 7.2.

Enzymatic reactions

$$E + H_2O_2 \Longleftrightarrow E_1 + H_2O \quad (1)$$

$$PhOH + E_1 \rightarrow PhO^{\cdot} + E_2 \quad (2)$$

$$AmNH_2 + E_1 \rightarrow AmNH^{\cdot} + E_2 \quad (2^{*})$$

$$PhOH + E_2 \rightarrow PhO^{\cdot} + E \quad (3)$$

$$AmNH_2 + E_2 \rightarrow AmNH^{\cdot} + E \quad (3^{*})$$

Nonenzymatic reactions

$$2\,PhO^{\cdot} \rightarrow P_1 \quad (4)$$

$$2\,AmNH^{\cdot} \rightarrow P_2 \quad (4^{*})$$

$$PhO^{\cdot} + AmNH^{\cdot} \rightarrow P_3\ (PhO\text{-}HNAm) \quad (4^{**})$$

$$PhO^{\cdot} + AmNH_2 \Longleftrightarrow PhOH + AmNH^{\cdot} \quad (5)$$

Scheme 1. Minimum reactions required to explain the kinetics of amine oxidation in the presence of phenolic compounds.

Phenols are much more likely to compete with TMB as inhibitors and/or alternative reducing substrates by binding in the HRP distal region (see [32] for a review of substrate binding sites on HRP). This would be consistent with the competitive inhibition observed for the oxidation of TMB in the presence of ANP, GA and po-lydisulfides poly(ADSNP) and poly(DSGA) (Figs. 4 and 6).

Polymer phenols are significantly more active inhibitors than their monomer analogs, i.e., an increase in the size of the polymer phenols does not weaken their binding to HRP.

Phenols and TMB are oxidized by Compounds I and II by reactions (2), (3) and (2*), (3*) with the production of phenoxyl and aminyl radicals, respectively. If the most reactive component is the phenol with $k_2 > k_2^{*}$ and $k_3 > k_3^{*}$, phenoxy radicals will initially dominate the product distribution and the phenol will be exhausted before the amine. The phenoxyl radicals can react with each other to give the dimeric product P_1 by reaction (4), or they can react with TMB by the exchange reaction (5). If the activity of $PhO^{\cdot}$ is sufficiently high, reaction (5) proceeds from left to the right generating TMB radicals with concomitant regeneration of phenol which may re-enter the peroxidase cycle. This variant of co-oxidation with the phenols DDS and poly(DSDDS), i.e., phenoxyl radicals of DDS and poly(DSDDS) are sufficiently active to oxidize TMB to the cation-radical $(TMB)^{\cdot +}$ and the recombination of the latter results in the product P_2 (reaction (4*))

$$(DDS)^{\cdot} + TMB \rightarrow DDS + (TMB)^{\cdot +}$$

If the amine is oxidized faster than the phenol, i.e., if at $[AmNH_2]_0 = [PhOH]_0$ $k^{*}_2 \gg k_2$ and $k_3^{*} \gg k_3$, the aminyl radicals generated cannot only recombine to give the product P_2 (reaction (4*)) but also oxidise the phenol by reaction (5*) with the production of phenoxyl radicals and regeneration of TMB

$$(TMB)^{\cdot +} + PhOH \rightarrow TMB + PhO^{\cdot} \quad (5^{*})$$

This variant of the TMB co-oxidation occurs in the presence of GA, ANP and their polydisulflde poly-(DSGA), poly(ADSNP) and other compounds studied previously [11,12]. With these phenolic substrates, reaction (5*) causes the induction period Δt (lag phase) in the formation of the oxidation product (P_2) and accounts for the observed competitive inhibition of TMB oxidation (Table 2).

The most effective inhibitor of the HRP-catalyzed oxidation of TMB is poly(DSGA) (K_i== 1.3 μM, f = 35.6). Monophenol ANP and its polydisulfide are less effective inhibitors (Table 2).

Table 3
Comparison of steady-state kinetic parameters (K_i,f) and pre-steady-state rate constants (k_2,k_3) for polydisulfides of phenols (pol) and analogs of their monomer units (mon)

Polydisulfides of phenols	N	α	α/n	α	α/n
		K_i(mon)/K_i(pol)		f(pol)/f(mon)	
Poly(DSGA)	~7.5	10.2	1.4	13.7	1.8
Poly(ADSNP)	~7	8.8	1.2	8.4	1.2
		k_2(pol)/k_2(mon)		k_3(pol)/k_3(mon)	
Poly(DSGA)	~7.5	10.3	1.4	16.0	2.1

In Table 3, a comparison is made of the steady-state kinetic parameters (K_i,f) and pre-steady state rate constants (k_2,k_3) for the polydisulfide derivatives of two phenols (pol) and their monomeric analogues (mon). The efficiency of poly(DSGA) as an inhibitor of TMB oxidation was 10.2 times higher than that of GA. Although the average number of monomers in this polid-isuffide is ~7,5, its antiradical activity exceeds that of GA by a factor 10.2 (not 7.5, as might be expected). If we divide the value $\alpha = k_i(mon)/k_i(pol)$ by the number of monomer's units in polymer molecule ***(n)***, we will obtain for poly(DSGA) the value (α /n) = 1.36; this can be considered as an arbitrary quantitative characteristic of the "intramolecular synergism" of poly(DSGA) as an antioxidant compared to the monomer form. The value of α/n for poly(DSGA), obtained from the ratio from f(pol) to/(mon) is 1.8.

"Intramolecular synergism" is also apparent from the ratios of the rate constants given in Table 1 for GA and poly(DSGA), i.e., k_2(pol)lk_2(mon) (α/n = 1.3) and k3(po1)/k3(mon) ((α/n = 2.1). Poly(DSGA) has previously been shown to exhibit "intramolecular synergism" as an inhibitor in the radical-generating system "ferritin-H_2O_2-TMB" [33]: $k_i(mon)/k_i(pol)$ = 11.2 and (α/n) = (11.2/7.5) = 1.5. The characteristics of "intramolecular synergism" for poly(ADSNP) with α/n = 1.2 (Table 3) are significantly less than those of poly (DSGA).

The phenomenon of "intramolecular synergism" for phenol-amine mixtures was first demonstrated and analyzed in terms of the exchange reaction (5) by Emanuel and co-workers [15,16,23]. Based on our previous data [1-13], the findings of the present work, and on the scheme of co-oxidation of "amine-phenol" pairs, we conclude that the general features of the HRP-catalyzed oxidation of such pairs depend the following factors:

1. The relative reactivity of the amines and phenols with respect to HRP Compounds I and II (i.e., the rate constants of reactions (2) and (2*), (3) and (3*)).
2. The initial and relative concentrations of PhOH and $ArNH_2$.
3. The reactivity of phenoxyl ($PhO^{\cdot}$) and aminyl ($ArNH^{\cdot}$) radicals in the exchange reaction (5), i.e., the relative and absolute values of the rate constants k_5 and k_5.
4. The steady-state concentrations of Compounds I and II in a particular HRP-catalyzed co-oxidation of "amine-phenol" pairs.
5. The reaction medium since we have shown in our earlier work with the pairs "TMB-ANP" and "TMB-poly(ADSNP)" [10]: in 10 mM phosphate buffer, pH 6.4 supplemented with 5% DMF, ANP and poly(ADSNP) inhibited the TMB oxidation, whereas in reversed micelles of aerosol OT (200 mM) in heptane containing 1 mM phosphate buffer, pH 6.4 with 1.5% DMF, the same compounds activated TMB oxidation ~2-fold (ANP) and -4-fold (poly(ADSNP).

The ability to predict the kinetic characteristics and product profile of HRP-catalyzed co-oxidations of "amine-phenol" pairs is important for developing new protocols for phenol analysis and immunoassay diagnostic kits. This paper has demonstrated the importance of the chemical reactions that occur between the free radical products of the first substrate oxidation and the second substrate referred to as an "exchange reaction". Moreover, a comparison of the data of this study in aqueous media with that previous reported in reversed micelles [10], shows that the direction of the exchange reac-

tion (5) can be reversed, at least for the TMB-ANP and TMB-poly(ADSNP) substrate pairs.

The present study has demonstrated that poly (DSGA) as well as polydisulfide of resorcinol [12] are highly effective inhibitors of HRP-catalyzed TMB oxidation and at relatively low concentration (μM) can be used to terminate the reaction. This is has considerable potential for commercial use in EIA, whose successful automation requires the development of safe, stable, easy to use but effective stopping-reagents for HRP-catalyzed reactions. The polydisulfides used in this study fulfill these criteria.

The substituted phenols and their polydisulfides with moderate K_i (10^{-5}-10^{-4} M) can be used to calibrate assays for the "total antioxidant ability" of biological fluids such as human blood serum.

5. Abbreviations

HRP	horseradish peroxidase isoenzyme C
ANP	2-amino-4-nitrophenol
GA	gallic acid
DDS	4,4′-dihydroxydiphenylsulfone
poly(ADSNP)	poly(2-aminodisulfide-4-nitrophenol)
poly(DSGA)	poly(disulfide of gallic acid)
poly(DSDDS)	poly(3-disulfide-4,4'-dihydroxydiphenylsulfone)
TMB	3,3',5,5' -tetramethylbenzidine
f	stoichiometric inhibition coefficient
K_i	inhibition constant
Δt	induction period
AmNH2	amines
InH	inhibitors
PhOH	phenols

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