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Reactions of "hybrid" Mn-peroxidase of the white rot fungus *Panus tigrinus* with benzylic alcohols in the presence of mediators

Alexander Lisov[a], Alexey Leontievsky[a,*], Ludmila Golovleva[a], Christine Evans[b]

[a] *G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow 142290, Russia*
[b] *School of Biosciences, University of Westminster, London W1W 6UW, UK*

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Abstract

"Hybrid" Mn-peroxidase (hMnP) isolated from the white rot fungus *Panus tigrinus* 8/18 was studied with respect to its reactions with veratryl alcohol in the presence of typical laccase mediators in the reaction mixture. Eight compounds were tested as potential mediators in this reaction and only 1-hydroxybenzotriazole (HBT) and 3-hydroxy-1,2,3-benzotriazin-4(3H)-one (HBTO) were found to be effective. Up to 99% of 1 M veratryl alcohol was oxidized with formation of veratraldehyde as a reaction product over 24 h depending on the buffer system used. Except for veratryl alcohol, anisyl alcohol but not benzyl alcohol was oxidized in this reaction. Reactions with the participance of mediators were not catalytic, and the mediators were consumed during reaction with formation of dehydroxylated derivatives. Reactions with both HBT and HBTO resulted in temporal inactivation of hMnP. Kinetics of hMnP inactivation revealed it to be a pseudo-third order reaction. Investigation of the transformation of the absorption spectra of hMnP redox cycle intermediates in the presence of HBT or HBTO showed that the most likely reason of hMnP inactivation was its interaction with non-oxidized mediators.

Keywords: *Panus tigrinus*; "Hybrid"; Mn-peroxidase; Mediators

1. Introduction

White rot fungi are the most effective lignin degraders in nature. A general conception of fungal biodegradation of lignin is the necessity of a primary attack on lignin by extracellular oxidoreductases—lignin peroxidase, Mn-peroxidase and laccase [1]. This list evidently should be supplemented with "classic" peroxidase, also produced by ligninolytic basidiomycetes [2]. A common feature of catalysis by these enzymes is the use of mediation effects. This supposes that a target substrate is oxidized not directly but by means of low-molecular mass intermediate compounds or mediators. The mediators may simply transfer the redox potential of the enzyme to the substrate, but also often promote oxidation of substrates possessing more positive redox potential than that of the enzyme itself.

This effect is most visible in the case of laccases with low redox potential compared to ligninolytic peroxidases. In contrast to lignin peroxidase, laccase is not able to oxidize nonphenolic substructures of polymeric lignin and its break-down products. However, in the presence of synthetic mediators, fungal laccases are capable of oxidation of these compounds as well as a number of other persistent chemicals, including xenobiotics [3,4]. Mediation effects under natural conditions are potentially possible, with syringaldehyde, a product of lignin degradation [5], 3-hydroxyanthranilic acid, a precursor of fruit body pigment in the basidiomycete *Pycnoporus cinnabarinus* [6], low-molecular mass thermolabile agents of *Trametes versicolor* [7], and a number of other

Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate); HAA, 3-hydroxyanthranilic acid; HBT, 1-hydroxybenzotriazole; HBTO, 3-hydroxy-1,2,3-benzotriazin-4(3H)-one; hMnP, hybrid Mn-peroxidase; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; VA, veratryl alcohol; VLA, violuric acid

* Corresponding author. Tel.: +7 95 925 7448; fax: +7 95 956 3370.
E-mail address: leont@ibpm.pushchino.ru (A. Leontievsky)

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compounds, produced by white rot fungi [4], all mentioned as laccase mediators.

In the case of lignin peroxidase, the most described mediator is veratryl alcohol [8]. Veratryl alcohol, as well as other similar compounds, is produced by a number of ligninolytic fungi simultaneously with lignin peroxidase secretion, during the lignin degradation process. [9,10]. The reaction scheme of Mn-peroxidase suggests the oxidation of Mn^{2+}, as a reducing substrate, with formation of Mn^{3+}. Newly formed and stabilized by an appropriate chelator, Mn^{3+} is assumed to be a mediator, capable of non-enzymatic reactions with target organic substrates at a distance from the enzyme, i.e. it is a carrier of oxidative potential of the enzyme [11]. Similarly to laccase, MnP, or more precisely, enzyme generated Mn^{3+}/chelator complex, is capable of oxidation of mainly low-potential phenolic compounds, but in the presence of thiols or unsaturated fatty acids, MnP is able also to oxidize persistent non-phenolic compounds. In this case, Mn^{3+}/chelator complex oxidizes thiols or lipids to thiol-radicals or lipid peroxy-radicals, which in turn, react with persistent target substrates, and are in fact, mediators [12–14]. The participation of thiols in the natural process of lignin degradation is unlikely [12], but action of unsaturated lipids seems to be more proven: their formation has been found during destruction of lignocellulose by ligninolytic fungi [15]. These processes have to be studied in more detail, however, their importance for development of technological approaches is obvious.

In spite of some differences in oxidation of similar substrates [16], both laccase and Mn-peroxidase belong to the "phenoloxidases" family of enzymes, oxidizing phenols and aromatic amines preferentially. The list of mediators extending the oxidative potential of laccase is substantial, and the effectiveness of different laccase mediators could be based on different molecular mechanisms [4,17]. The mediators well known for Mn-peroxidases are usually restricted to thiols and unsaturated lipids. In addition, successful application of 3-hydroxy-1,2,3-benzotriazin-4(3H)-one (HBTO), a known laccase mediator [18], has been described for paper pulp bleaching by Mn-peroxidase [19]. Another laccase mediator 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) has been used for veratryl alcohol oxidation by plant peroxidases from horseradish [20] and tobacco [21].

The aim of this work was to study the effects of different laccase mediators on reactions of hybrid Mn-peroxidase of *P. tigrinus* with aromatic alcohols that were not substrates for phenoloxidases.

2. Experimental procedures

2.1. Materials

All substrates, mediators and solvents were commercially available from Sigma, Aldrich and Fluka. All components of culture medium for cultivation of the fungus were obtained from Reakhim (Russia).

2.2. Enzyme preparation

Hybrid Mn-peroxidase (hMnP) was purified from submerged culture of *Panus tigrinus* 8/18 as described earlier [22]. The purified enzyme was electrophoretically homogeneous and had molecular mass of 43 kDa and Rz ($A_{406/280}$) = 4.0.

2.3. Enzyme assay

During cultivation of the fungus, in purification of hMnP and in reactions with mediators, the hMnP activity was evaluated spectrophotometrically by the rate of formation of Mn^{3+}/malonate complex at 270 nm, ε_{270} = 11.69 mM^{-1} cm^{-1} [23]. Reaction mixture content: 50 mM Na-malonate buffer, pH 4.5; $MnSO_4$, 1 mM; H_2O_2, 0.1 mM. The amount of enzyme converting 1 μmol of substrate or generating 1 μmol of Mn^{3+}/malonate over 1 min was taken as one unit (U) of activity.

When the rate of inactivation of hMnP was studied, hMnP activity was detected by the rate of 2,6-dimethoxyphenol oxidation. The increase of absorption at 469 nm was followed. Reaction mixture content: 20 mM Na-tartrate buffer, pH 4.0; 2,6-dimethoxyphenol, 1.5 mM; $MnSO_4$, 1.0 mM; H_2O_2, 0.1 mM.

2.4. Reactions with participance of mediators

Reactions of hMnP with aromatic alcohols were held for 24 h at 30 °C in reaction mixtures containing aromatic alcohol, 1 mM; $MnSO_4$, 1 mM; H_2O_2, 0.1 mM; mediator, 5 mM; hMnP, 10 U; and 50 mM Na-acetate, Na-tartrate, or Na-malonate buffers, pH 4.5. Veratryl (3,4-dimethoxybenzylic) alcohol, anisyl (4-methoxybenzylic) and benzylic alcohols were used as target substrates. Potential mediators used were 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate), 1-hydroxybenzotriazole (HBT), 3-hydroxy-1,2,3-benzotriazin-4(3H)-one, 3-hydroxyanthranilic acid (HAA), 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO), 4-OH-TEMPO, violuric acid (VLA), catechol, guaiacol and 2,4-dihydroxyphenylalanine. The mediators were added to reaction mixtures in methanol solution, except HBTO that was dissolved in dimethylformamide.

Reactions of Mn^{3+}/acetate with veratryl alcohol (VA) were conducted under the same conditions with replacement of $MnSO_4$, H_2O_2 and hMnP with 1 mM Mn^{3+}/acetate.

Quantitative analysis of the decrease in benzyl alcohols and formation of their corresponding aldehydes was conducted using an HPLC system (Waters, USA) with a reversed phase column ODS-2 Supelco C_{18} 250 mm × 4.6 mm. Elution was carried out with an increasing gradient of methanol in water 20–100% for 20 min at 55 °C. Detection was at 254 nm using a UV detector. Benzylic

alcohols and aldehydes were identified by the retention time determined for corresponding standards. Benzotriazole and benzotriazine, the products of dehydroxylation of HBT and HBTO correspondingly, were identified by mass-spectrometry (Finnigan Mat 8430, Germany) operated at an ionization energy of 70 ev with direct evaporation of sample. Compounds for MS analysis were purified by HPLC.

Stoichiometry of veratraldehyde formation depending on HBT and HBTO concentration in reactions of Mn^{3+}/acetate with VA was determined for 24 h at 30 °C in reaction mixtures containing 50 mM Na-acetate or Na-malonate buffers, pH 4.5, Mn^{3+}/acetate, 5 mM; veratryl alcohol, 5 mM; HBT or HBTO at a concentration range of 30–150 μM.

2.5. hMnP inactivation

hMnP inactivation was studied at 30 °C in 50 mM Na-malonate buffer, pH 4.5, containing hMnP, 0.45 μM; mediators, 5–60 mM; VA, 1 mM; H_2O_2, 0.1 mM; $MnSO_4$, 1 mM. hMnP activity was expressed as a ratio of enzyme activity at the moment of time t (A) to the initial activity (A_0).

2.6. Intermediates of catalytic cycle of hMnP

Absorption spectra of the intermediates of the hMnP redox cycle were recorded in 50 mM Na-malonate buffer, pH 4.5 at room temperature using a spectrophotometer (Shimadzu UV-2501 PC, Japan). The native form of hMnP had absorption maxima at 406, 504 and 638 nm. Compound II was prepared by the addition of 10 mole equivalents of H_2O_2 to the native enzyme and had absorption maxima at 420, 530 and 558 [22]. hMnP Compound III was prepared by treatment of native enzyme with 300 mole equivalents of H_2O_2 for 2 min with subsequent removal of excess H_2O_2 by 200 U/ml of catalase for 5 min.

2.7. Analytical methods

hMnP concentration was determined, using a value of coefficient of molar extinction at 406 nm (ε_{406}) equal to 131 $mM^{-1}\,cm^{-1}$. The coefficient of molar extinction at 406 nm was determined by the pyridine hemochrome method [24].

Determination of H_2O_2 concentration was based on absorption at 240 nm, ε_{240} = 39.6 $mM^{-1}cm^{-1}$ [25].

Methanol solutions of Mn^{3+}/acetate were prepared directly before use. Insoluble particles were removed by centrifugation. Concentration of Mn^{3+} was determined by ferricyanide oxidation, ε_{420} = 1.02 $mM^{-1}\,cm^{-1}$ [26].

3. Results

3.1. Reactions of hMnP with benzylic alcohols with participance of mediators

Veratryl alcohol was used as a test substrate to check the effectivity of mediators, as a non-phenolic aromatic compound not oxidized by MnP or Mn^{3+}/chelator system [27]. As potential mediators for hMnP (*P. tigrinus*), compounds known as synthetic mediators for fungal laccases ABTS, TEMPO, 4-OH-TEMPO, VLA, HBT and HBTO [17,18] were used, with 3-hydroxyanthranilic (HAA) acid as a natural laccase mediator [6], and catechol, guaiacol and 3,4-dimethoxyphenylalanine as typical phenols and aminoacids, with capability as mediators in laccase reactions [4]. These reactions were monitored in 20 mM Na-malonate, Na-tartrate and Na-acetate buffers, pH 5.0.

hMnP (*P. tigrinus*) isolated from submerged culture did not oxidize VA in the absence of mediators in the reaction mixture(Table 1). Of the list of compounds tested, only HBT, HBTO and VLA revealed properties as mediators in reaction

Table 1
Oxidation of aromatic alcohols by hMnP of *P. tigrinus* with participance of mediators

Content of reaction mixture	Yield of veratraldehyde (%)		
	Na-malonate	Na-tartrate	Na-acetate
hMnP ($+H_2O_2 + Mn^{2+}$), no mediators + veratryl alcohol	0	0	0
hMnP ($+H_2O_2 + Mn^{2+}$) + HBT + veratryl alcohol	95	95	48
hMnP, no Mn^{2+} ($+H_2O_2$) + HBT + veratryl alcohol	0	0	5
hMnP + HBTO + veratryl alcohol	90	90	99
hMnP, no Mn^{2+} + HBTO + veratryl alcohol	0	0	6
hMnP + VLA + veratryl alcohol	0	15	14
hMnP, no Mn^{2+} + VLA + veratryl alcohol	0	0	2
hMnP ($+H_2O_2 + Mn^{2+}$) + HBT + anisyl alcohol	60	66	35
hMnP ($+H_2O_2 + Mn^{2+}$) + HBTO + anisyl alcohol	61	62	85
hMnP ($+H_2O_2 + Mn^{2+}$) + HBT + benzyl alcohol	0	0	0
hMnP ($+H_2O_2 + Mn^{2+}$) + HBTO + benzyl alcohol	0	0	0
Mn^{3+}/acetate + veratryl alcohol	0	0	0
Mn^{3+}/acetate + HBTO + veratryl alcohol	35	34	37
Mn^{3+}/acetate + HBT + veratryl alcohol	34	30	32
Mn^{3+}/acetate + VLA + veratryl alcohol	0	3	9

Reaction of hMnP: hMnP, 10 U; H_2O_2, 0.1 mM; $MnSO_4$, 1 mM; mediator (HBT, HBTO or VLA), 5 mM; aromatic alcohol, 1 mM; 50 mM buffer, pH 4.5; 24 h at 30 °C. Reaction of Mn^{3+}/acetate: Mn^{3+}, 1 mM; mediator, 5 mM; veratryl alcohol, 1 mM; 50 mM buffer, pH 4.5; 24 h at 30 °C.

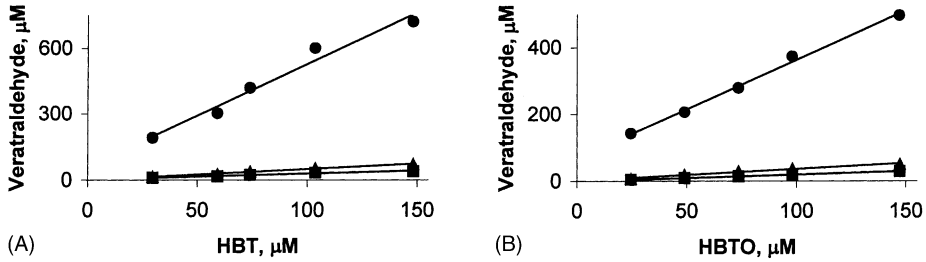

Fig. 1. Stoichiometry of veratraldehyde formation in reactions of Mn^{3+}/acetate with veratryl alcohol and mediators. (A) HBT, (B) HBTO: (●) 50 mM Na-acetate buffer, pH 4.5; (■) 50 mM Na-malonate buffer, pH 4.5; (▲) 50 mM tartrate buffer, pH 4.5. Concentration of both veratryl alcohol and Mn^{3+}/acetate was 5 mM.

of hMnP (*P. tigrinus*) with VA under the presented conditions. During 24 h of reaction, up to 99% of VA as a target substrate was oxidized, depending on the type of buffer system used. Besides VA, in the presence of HBT or HBTO, hMnP also oxidize anisyl alcohal, but not benzyl alcohol.

In the absence of Mn^{2+}, hMnP did not oxidized VA, with the exception of reactions in acetate buffer where up to 6% of veratraldehyde was found as a reaction product. This gave reason to check the possibility of VA oxidation by the artificial Mn^{3+}/chelator complex. It was found that Mn^{3+}/acetate was capable of replacing the system hMn-P/H_2O_2/Mn/chelator in this reaction with oxidation of about 35% of VA with participance of HBT and HBTO, and up to 9% with VLA (Table 1). Under the conditions of excess concentration of VA and Mn^{3+}/acetate, the amount of veratraldehyde formed had a linear dependence on the amount of HBT or HBTO added to the reaction mixture (Fig. 1). The low stoichiometric ratio of veratraldehyde: mediator, equal to ∼1.5:1 (malonate, tartrate buffers) or 4:1 (acetate buffer), suggested the absence of a cyclic process of oxidation–reduction of the mediators and consumption of mediators during the reaction. From the reaction mixtures, the dehydroxylated derivatives of HBT or HBTO–benzotriazole and benzotriazine, respectively, were isolated by HPLC and identified by mass-spectrometry.

3.2. hMnP inactivation in the presence of mediators

During reaction of hMnP (hMnP/H_2O_2/Mn/malonate system) with VA in the presence of HBT as mediator, a temporal decline in enzyme activity was found. Over 3.3 h of reaction time, hMnP activity decreased by about 50% (Fig. 2). The enzyme activity value was not changed after incubation of hMnP in malonate buffer at 30 °C over the same time.

The inactivation of hMnP occurred also during incubation of the enzyme in the presence of hydrogen peroxide and without substrate (Fig. 3). However, the presence in the reaction mixture of Mn^{2+}, HBT or HBTO, protected hMnP from inactivation by hydrogen peroxide. Mn^{2+} is a natural substrate of hMnP capable of reducing the intermediates of the hMnP redox cycle. The protective effect of HBT and HBTO indicated that these compounds were also reducing substrates of the enzyme. This was confirmed by reduction of compound II, a one electron oxidized intermediate of the hMnP redox

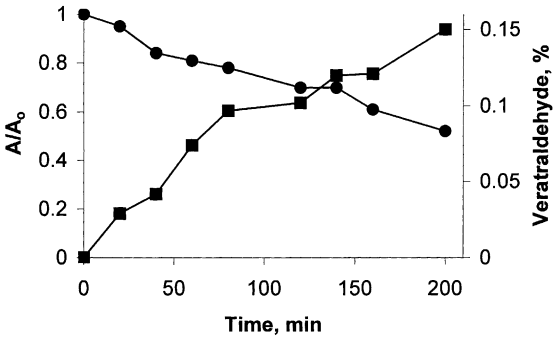

Fig. 2. Inactivation of hMnP during oxidation of veratryl alcohol with HBT. (■) Yield of veratraldehyde and (●) relative activity of hMnP. Concentration of: VA, 1 mM and HBT, 5 mM.

cycle, with formation of the native form of the enzyme, by the action of HBT or HBTO (Fig. 4). VA did not protect hMnP from inactivation under these conditions.

Inactivation of the peroxidase by hydrogen peroxide occurred with formation of catalytically non-active compound III with subsequent heme bleaching. Compound III of fungal Mn-peroxidase was shown to be able to revert to native form with Mn^{2+} as a substrate, and Mn^{3+} as a product of the reaction [26]. We have confirmed the effect of HBT as a reducing substrate for compound III of hMnP.

Chemically prepared compound III of hMnP (*P. tigrinus*) had absorption maxima at 418, 542 and 580 nm, the intensity

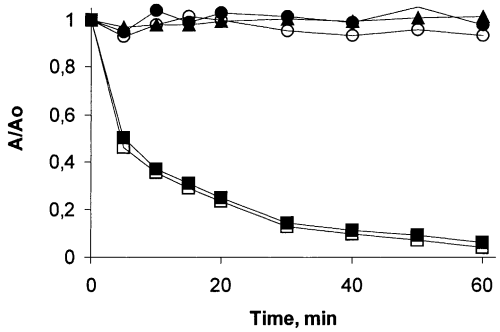

Fig. 3. Effect of hydrogen peroxide on hMnP activity. Relative activity of hMnP during incubation with H_2O_2 only (□), and with additional presence of Mn^{2+} (▲), HBT (●), HBTO (○), or veratryl alcohol (■). Content of reaction mixture: 50 mM Na-malonate, pH 4.5; hMnP, 0.45 μM; H_2O_2, 0.1 mM and $MnSO_4$, 0.1 mM; HBT, 5 mM; HBTO, 5 mM or veratryl alcohol, 1.0 mM.

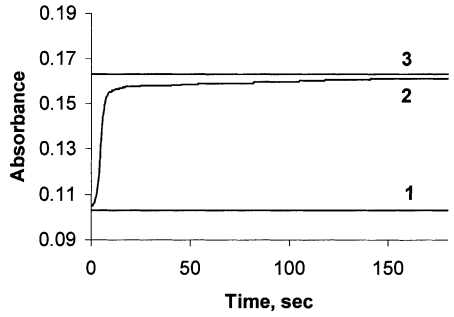

Fig. 4. Reduction of hMnP compound II to native form. (1) Absorption at 406 nm of one electron oxidized intermediate of hMnP redox cycle compound II; (2) change in absorption at 406 nm after addition of 10 mole equivalents of HBT to hMnP compound II; (3) Absorption at 406 nm of native enzyme: 50 mM Na-malonate buffer, pH 4.5; hMnP concentration, 1.3 μM.

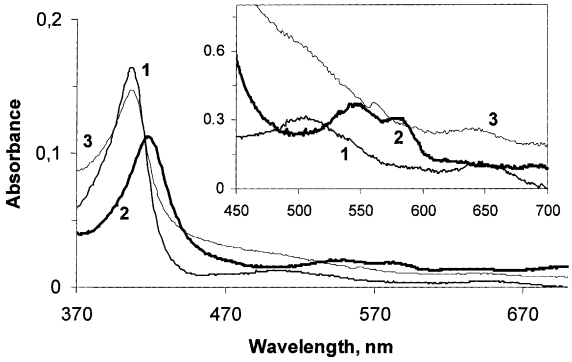

Fig. 5. Reactivation of compound III of hMnP by HBT. Absorption spectra of native form of hMnP (1), compound III (2) and native hMnP reactivated by addition of HBT to compound III (3). Concentration of: hMnP, 2.1 μM; HBT, 5 mM.

of absorption of the Soret band was 50% of that of the native enzyme (Fig. 5). These characteristics were similar to that of MnP of *Phanerochaete chrysosporium* [28]. With excess H_2O_2, an irreversible destruction of hMnP (*P. tigrinus*) compound III occurred with disappearance of absorption in all ranges of the spectrum. When excess H_2O_2 was removed by catalase, compound III was stable for 15 min, and then spontaneously transformed to native enzyme during 1 h (data not shown). After addition of HBT to the stable compound III, the latter immediately reverted to native form with recovery of the characteristic absorption maxima at 406, 504 and 638 nm (Fig. 5). However, when the intensity of absorption at 406 nm was at 80% of the initial intensity, the absorption at 504 and 638 nm was 50% higher compared to native enzyme.

The addition of VA had no effect on compound III transformation. Analogous experiments with HBTO were not done due to its strong absorption in the visible range of the spectrum.

The decrease in absorption of the Soret band in the case of native hMnP reverted from compound III by HBT could be the result of partial decolouration of heme because of the temporal effect at high concentrations of H_2O_2. Such effects were seen in the spectrum of native hMnP formed by spontaneous reversion of compound III. However, in this case, no increase in the absorption in the visible region was observed. Therefore, we suggest that the alteration in the ratio of intensity of absorption in the spectrum of native enzyme reverted from compound III by addition of HBT, could result from the interaction of hMnP with the mediator or with its oxidation product.

The mediators HBT and HBTO were capable of inactivating hMnP, and the rate of inactivation was accelerated with increasing concentration of mediator. hMnP inactivation was studied under the condition of pseudo-first order reaction with excess concentration of inactivator. The linearity of the kinetic curves reflected semi-logarithmic conversions of hMnP inactivation in the presence of different concentrations of HBT, according to the equation of pseudo-first order reactions (Eq. (1), [29], Fig. 6A):

$$\ln\left(\frac{A}{A_0}\right) = -\ln kt + \ln(A_0) \quad (1)$$

where A_0 is the initial activity of the enzyme and A is the activity of the enzyme at time t.

The slopes of these conversions were used to calculate the k_{app} of the pseudo-first order reaction. The resultant k_{app} values did not show a linear relationship with the concentration of inactivator, but had linear dependence on the HBT concentration being raised to the third power, with curving of the

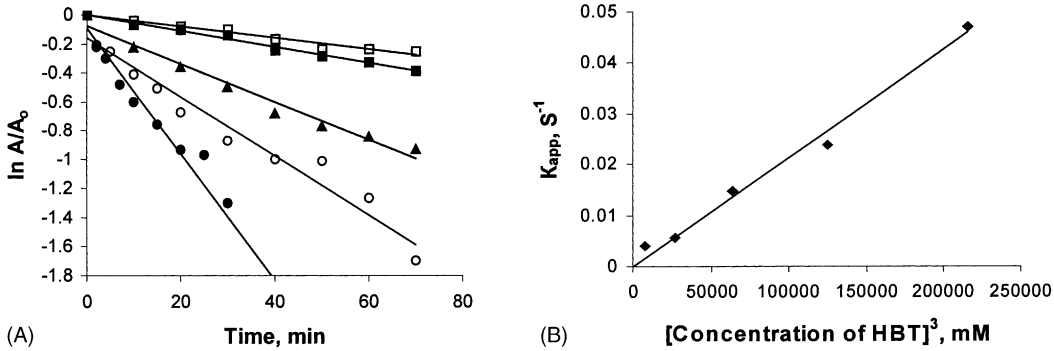

Fig. 6. Kinetics of inactivation of hMnP by HBT. (A) Semilogarithmic plot of kinetic curves of hMnP inactivation in the presence of different concentration of HBT. HBT concentrations: (□) 20 mM; (■) 30 mM; (▲) 40 mM; (◯) 50 mM and (●) 60 mM. (B) Dependence of k_{app} from HBT concentration.

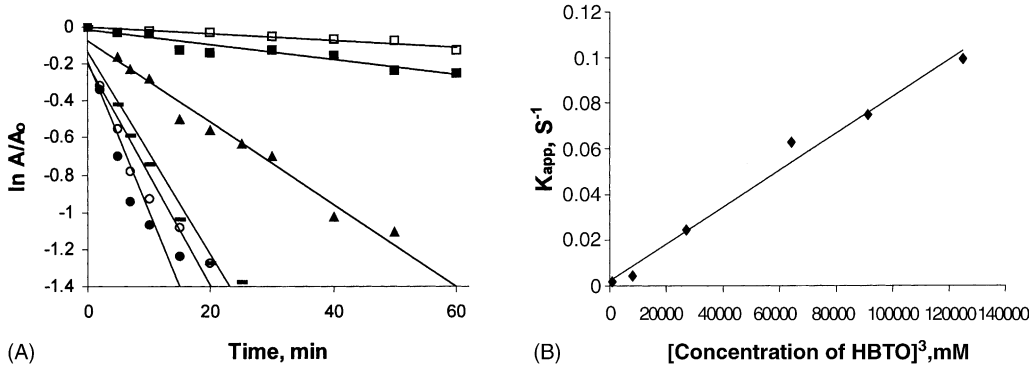

Fig. 7. Kinetics of inactivation of hMnP by HBTO. (A) Semilogarithmic plot of kinetic curves of hMnP inactivation at the presence of different concentration of HBTO. HBTO concentrations: (□) 10 mM; (■) 20 mM; (▲) 30 mM; (-) 40 mM; (◯) 45 mM and (●) 50 mM. (B) Dependence of k_{app} from HBTO concentration.

linear approximation through zero (Fig. 6B). This means that inactivation of hMnP by HBT is by a pseudo-third order reaction caused by the inactivator. The analogous experiments with HBTO obtained similar results (Fig. 7A and B).

Inactivation of hMnP by HBT (in the absence of Mn and H_2O_2) was followed by a decrease in intensity of absorption of the Soret band and an increase of absorption in the visible region of the enzyme spectrum (Fig. 8). This transformation of the hMnP absorption spectrum occurred under the described conditions over 2 h. Afterwards, the intensity of absorbance of the Soret band decreased to 50% (the maximum moved from 406 to 403 nm) and in the visible region of the spectrum at 504 and 638 nm, it was increased 30% compared with the native form of hMnP. Consequently, the results of both experiments, with complete reaction mixture (Fig. 4) and with HBT only (Fig. 8), indicated that the reason for hMnP inactivation could be its interaction with mediators or with the products of HBT or HBTO oxidation.

To clarify this, we studied the effect of different concentrations of VA on the rate of hMnP inactivation. If the hMnP inactivator was the product of oxidation of the mediator, an increase in VA concentration should strengthen the interaction between hMnP and VA with the (radical?) product of oxidation of the mediator that, in turn, would result in decreasing the rate of enzyme inactivation. However, VA in the range of concentration 1–30 mM did not protect hMnP from inactivation (data not shown). This result indicates that the inactivation of hMnP is not caused by the mediator oxidation product.

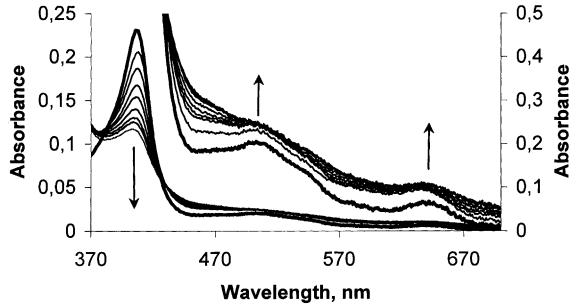

Fig. 8. Transformation of absorption spectrum of hMnP after addition of HBT. Thick line, initial spectrum of native enzyme. The spectra were recorded after addition of HBT and then, each 10 min. Concentration of: hMnP, 2.1 μM; HBT, 5 mM.

4. Discussion

4.1. Reactions of hMnP with benzylic alcohols with the participance of mediators

Mn-peroxidase of *P. tigrinus* 8/18 belongs to the family of hybrid or polyvalent Mn-peroxidases capable of completing its catalytic cycle (i.e. to reduce one electron oxidized compound II to native form) both by oxidation of Mn^{2+} and by oxidation of organic substrates, such as phenols and aromatic amines [22]. Such enzymes have been isolated from cultures of other ligninolytic fungi also [30,31].

Previously we isolated hMnP from solid-state cultures of *P. tigrinus*, grown on wheat straw [32]. In the presence of Mn^{2+} this enzyme was capable of oxidising veratryl alcohol to veratraldehyde, and of splitting the inter-monomeric linkage of a non-phenolic dimeric model compound of lignin [32,33]. The hMnP isolated from liquid medium was not able to oxidize VA under similar conditions (Table 1). In spite of the similarity of the molecular masses (43 kDa) and absorption spectra of the hMnPs obtained from solid-state and submerged cultures of *P. tigrinus*, the reason for these differences in activity could be the isolation of different isoforms of the enzyme. Expression of both forms of typical and hybrid MnP has been described for *Pleurotus ostreatus* [31].

The compounds tested in this work as potential mediators for hMnP (*P. tigrinus*) are well known as effective mediators of fungal laccases. However, only HBT and HBTO, and to a lesser degree VLA, were effective mediators in reactions with hMnP (P. *tigrinus*) (Table 1). A similar set of laccase mediators, including ABTS, VLA, HBT, HBTO and HAA have been used for bleaching of paper pulp by MnP of the white rot fungus *T. versicolor*, and only HBTO showed a positive effect [19]. A general principle of mediator action supposes the sequence of reactions where firstly the mediator is oxidized

by laccase, and then the reactive product of oxidation of the mediator oxidizes the target substrate (in our case, benzylic alcohols) [17,34]. In the case of R–NOH compounds, such as HBT, VLA and HBTO, the reactive product of oxidation of the mediator is R–N(O)$^{\cdot}$ [35–37]. Because of the hybrid nature of hMnP of P. *tigrinus*, we have examined the possibility of VA oxidation in the absence of Mn^{2+}, suggesting that the mediators could be oxidized as organic substrates, alternative to Mn^{2+}. In this case, hMnP oxidized the effective mediators HBT and HBTO as well as ineffective ones such as ABTS (data not shown). However, the oxidized products of both HBT and ABTS did not oxidize VA as a target substrate under these conditions (Table 1). If one disregards the insignificant rate of VA oxidation in the absence of Mn^{2+} in acetate buffer, the oxidation of VA was detected mostly from hMnP in the presence of $Mn^{2+}$and H_2O_2 or by the artificial Mn^{3+}/acetate complex. This indicated that oxidation of the mediator to a reactive product was only possible in the presence of Mn^{3+}.

According to data from Hofrichter et al. [38], Mn^{3+} in the presence of oxygen is able to react with malonate and other α-hydroxyacids frequently used as chelators for stabilization of Mn^{3+} in aqueous medium. This interaction results in destruction of the chelators with concomitant formation of organic and inorganic radical products and H_2O_2. In our experiments with VA oxidation, these kinds of reactions could result in both an increase of oxidative potential of the reaction mixture and in the yield of veratraldehyde, or decrease the yield because the radical products of oxidation by the mediators have been “extinguished”. The ratio of the results of these processes could be determined by the structures of chelators, mediators and products of oxidation. This may explain the non-effectivity of several laccase mediators (e.g. ABTS) and the different yields of veratraldehyde when different buffer systems were used (Table 1). A low stoichiometric ratio of veratraldehyde: mediator suggested that the reaction of HBT or HBTO with VA was not cyclic, that the mediator was not a catalyst for this reaction, and that the product of interaction between mediator and VA was not reduced back to HBT or HBTO. Reaction of laccase with HBT was also non-cyclic, in the case of interaction of the laccase/HBT system with the target substrate in the reaction mixture when benzotriazole accumulated as a product of HBT oxidation [34].

Inactivation of laccase during reaction with HBT as mediator is well documented [34]. This was explained by covalent binding of the radical product of HBT oxidation with laccase protein, or even by co-binding of two or more laccase molecules with the product [39]. The inactivation of hMnP of *P. tigrinus* under analogous conditions (Fig. 2) cannot be explained by its thermo-inactivation as the enzyme was stable out of the reaction mixture. Inactivation of Mn-peroxidase by H_2O_2 with formation of compound III is well known, as is the protective effect of reducing substrates [26,28,40]. In the case of hMnP (*P. tigrinus*) inactivation of the enzyme could not be caused by H_2O_2 action as the enzyme was protected by reducing substrates Mn^{2+}, HBT and HBTO (Figs. 3 and 4) and probably by Mn^{3+}, a product of the reaction [26].

The decrease in intensity of absorption of the Soret band of hMnP reverted from compound III could be a consequence of partial decolouration of heme after contact with H_2O_2 at high concentration (Fig. 5). HBT and HBTO were able to inactivate the hMnP without H_2O_2 and $MnSO_4$ being in the reaction mixture. This suggests that, in contrast to laccases, hMnP was not inactivated by the oxidized mediators. The interaction of HBT with hMnP resulted in an increase in absorption at 504 and 638 nm maxima of the spectrum (Figs. 5 and 8). Since no such alteration was found in the spectrum of native hMnP reverted from compound III, this could be a consequence of interaction of hMnP with the mediator. Since the kinetics of inactivation of hMnP was of pseudo-third order reaction (Figs. 6 and 7), we suggest that hMnP inactivation may be the result of interaction with more than one molecule of mediator.

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