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Hybrid Mn-Peroxidase from the Ligninolytic Fungus *Panus tigrinus* 8/18. Isolation, Substrate Specificity, and Catalytic Cycle

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Abstract—Increased manganese concentration during submerged cultivation of the ligninolytic white rot fungus *Panus tigrinus* 8/18 on N-limited mineral medium resulted in the induction of Mn-peroxidase and laccase. The Mn-peroxidase was purified with the purity factor RZ (A_{406}/A_{280}) = 4.3. The purified enzyme catalyzed H_2O_2-dependent oxidation of phenol oxidase substrates (aromatic amines, 2,2′-azinobis-(3-ethylbenzthiazolinesulfonic acid), hydroquinone, 2,6-dimethoxyphenol) without Mn^{2+}, which is not typical for the usual Mn-peroxidases. Guaiacol and 2,4,6-trichlorophenol were not oxidized in the absence of Mn^{2+}. Study of absorption spectra of the intermediates of the catalytic cycle revealed that this peroxidase is able to complete the redox cycle, reducing one-electron oxidized intermediate (Compound II) by Mn^{2+}, as well as by an organic substrate (hydroquinone). This means that the enzyme is a "hybrid" Mn-peroxidase, different from the common Mn-peroxidases from ligninolytic fungi.

Key words: *Panus tigrinus*, Mn-peroxidase, hybrid peroxidase, absorption spectra, catalytic cycle

Lignin is one of the most abundant materials in the biosphere. This polymeric water-insoluble compound comprises up to ~25% of plant cell walls and is characterized by its resistance to both chemical and biological degradation. The most efficient degraders of lignin in nature are wood decay fungi (Basidiomycetes), which induce white rot of wood. These fungi contain extracellular oxidoreductases involved in lignin degradation, namely lignin peroxidase, Mn-peroxidase, and laccase.

The white rot fungus *Panus tigrinus* 8/18 belongs to a class of ligninolytic fungi that do not produce lignin peroxidase under any cultivation conditions. Mn-peroxidase and laccase activities were detected in the process of lignin degradation by this fungus [1, 2].

Typical Mn-peroxidase from the most studied white rot fungus *Phanerochaete chrysosporium* oxidized phenol oxidase substrates (phenols, aromatic amines, etc.) only in the presence of Mn^{2+} [3, 4]. Mn-peroxidase from *P. chrysosporium* has a characteristic peroxidase catalytic cycle, including oxidation of the native enzyme by hydrogen peroxide with the formation of Compound I; reduction of Compound I to Compound II by the reducing substrate (organic electron donor or Mn^{2+}); and final reduction of Compound II to the native enzyme mediated by Mn^{2+} [5, 6]. The product of Mn^{2+} oxidation is Mn^{3+}. It is a powerful oxidizer that can form chelate complexes with organic acids produced by the fungus in the process of lignin degradation. Mn^{3+} complexes are able to non-enzymatically oxidize organic compounds including lignin phenol substructures [7].

Mn-peroxidase of *P. tigrinus* 8/18 was earlier extracted by us from a solid-state culture grown on wheat straw and then characterized. This enzyme catalyzes H_2O_2- and Mn^{2+}-dependent oxidation of substrates that are specific for the typical Mn-peroxidase: syringaldazine, vanillylacetone, phenol red. However, unlike *P. chrysosporium* Mn-peroxidase, *P. tigrinus* Mn-peroxidase is able to oxidize phenolic substrates in the absence of Mn^{2+} analogous to horseradish peroxidase or *P. chrysosporium* lignin peroxidase. In the final reaction of the catalytic cycle the Compound II of *P. tigrinus*, Mn-peroxidase is reduced by both Mn^{2+} and an organic substrate (diaminobenzidine) [8, 9]. Furthermore, in the absence of any mediators this enzyme oxidized a non-phenolic dimeric model compound (β1 type lignin), and also depolymerized Bjorkman birch wood lignin [10]. The unusual properties of this enzyme suggested that the described *P. tigrinus* peroxidase has both lignin peroxidase and Mn-peroxidase activities and is in

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fact a manganese-dependent lignin peroxidase. Afterwards, analogous enzymes called hybrid or polyvalent peroxidases were also found in other ligninolytic fungi [11-14].

The purpose of this work was to isolate the hybrid peroxidase from submerged culture of *P. tigrinus* 8/18 and to characterize its catalytic properties.

MATERIALS AND METHODS

Materials. Hydroquinone (Reakhim, Russia), H_2O_2 (Mosreaktiv, Russia), malonate and guaiacol (Fluka, Germany), 2,2′-azinobis-(3-ethylbenzthiazolinesulfonic acid) (ABTS), 2,4,6-trichlorophenol (Sigma, USA), 2,6-dimethoxyphenol (Aldrich, USA), *o*-dianisidine and *p*-phenylenediamine (Serva, Germany) were used in this work. Other chemically pure reagents were of domestic origin (Reakhim, Russia).

Microorganism and cultivation conditions. This work uses the white rot Basidiomycete *P. tigrinus* 8/18 from the collection of the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (Pushchino, Russia). The inoculum was cultivated on soy-glycerol medium at 29°C for 5 days; before the inoculation, mycelium cultures were homogenized by mixing with porcelain beads [1]. The submerged culture was cultivated on mineral medium with low nitrogen concentration [15] containing 1% glucose and 0.05% Tween 80 in 750 ml flasks containing 200 ml of medium at 29°C on a shaker at 200 rpm. For the induction of hybrid Mn-peroxidase, $MnSO_4 \cdot H_2O$ was added to the medium. Since the salt is hygroscopic, the concentration of Mn^{2+} was determined using a Perkin-Elmer 5100 atomic absorption analyzer (USA). The media had manganese concentrations of 0.32 mg/liter (base medium) and 71 mg/liter [16].

Enzyme purification. Culture broth was separated from the mycelium by filtration through lavsan, and the pH value of the filtrate was adjusted to 5.0 with 1 M NaOH. The first stage of the purification was performed on TEAE-Servacell 23 (Reanal, Hungary). The ion exchanger, equilibrated with 20 mM sodium acetate buffer, pH 5.0 (buffer A), was introduced into the culture broth supernatant and kept overnight at 4°C. Then, the carrier with adsorbed enzyme was placed in a column (2.6 × 70 cm) and eluted with 0.5 M NaCl in buffer A. The active fractions were collected and concentrated by ultrafiltration on an Amincon PM-10 membrane (Millipore, USA) to 4.5-ml volume. The product was then gel filtered on a column (2.6 × 100 cm) with Sephadex G-100 (Pharmacia, Sweden), elution rate 18 ml/h, fraction volume 2 ml, working buffer A with 0.1 M NaCl. Fractions containing Mn-peroxidase activity but not laccase activity were pooled and then concentrated by ultrafiltration. Then anion-exchange chromatography on a Mono Q HR 5/5 column (Pharmacia) was performed. For this purpose, the enzymatic product was diluted 3 times with buffer A after the gel filtration and then placed on a column equilibrated with the same buffer. The elution was performed by a NaCl gradient in buffer A at flow rate of 1 ml/min. The final purification step was gel filtration on a Sephacryl S-100 HiPrep 16/60 column (Pharmacia), elution rate 0.2 ml/min, and fraction volume 0.5 ml. The active fractions were pooled and concentrated by ultrafiltration. If necessary, the enzymatic product underwent a second gel filtration under the same conditions. The purified product of hybrid Mn-peroxidase was concentrated and washed in bidistilled water to remove salt residues using an Amicon Centriprep YM-10 ultrafiltration tubes (Millipore).

Determination of enzyme activity. Laccase activity was determined by ABTS oxidation rate. The reaction was performed in buffer A containing 0.2 mM ABTS; the increase in absorbance was measured at 436 nm.

Hybrid Mn-peroxidase activity was determined by the rate of Mn^{3+}–malonate complex formation during the oxidation of Mn^{2+} [7]. The composition of the reaction mixture was 50 mM sodium malonate, pH 4.5, 1 mM $MnSO_4$, 0.1 mM H_2O_2. Increase in absorption was measured at 270 nm.

To determine the substrate specificity of the hybrid Mn-peroxidase, the reaction was performed in 20 mM sodium tartrate buffer, pH 4.0, with 0.1 mM H_2O_2 and 0.5 mM substrate. If necessary, 1 mM of $MnSO_4$ was also added. The following substrates were used: ABTS at 436 nm; 2,6-dimethoxyphenol at 469 nm; *p*-phenylenediamine at 320 nm; *o*-dianisidine at 470 nm; guaiacol at 470 nm; 2,4,6-trichlorophenol at 255 nm; hydroquinone at 254 nm. Rate of absorbance increase was measured at the corresponding wavelength.

The oxidase reaction of hybrid Mn-peroxidase was determined based on NADH oxidation rate with reduction of oxygen to H_2O_2 [3]. The content of reaction mixture was: 20 mM sodium-tartrate buffer, pH 4.0, 0.3 mM NADH, 0.1 mM $MnSO_4$. Decrease in absorbance was measured at 340 nm.

Enzyme activity was expressed in arbitrary units. One arbitrary unit (U) corresponded to the change in absorbance at a certain wavelength by 1 absorbance unit per 1 min. The specific activity was expressed in U/ml for culture broth or in U/mg for protein.

Absorption spectra. The absorption spectra of hybrid Mn-peroxidase were recorded using a Shimadzu UV-2501PC spectrophotometer (Japan) at protein concentration 0.1-1.0 mg/ml in bidistilled water at room temperature. The intermediates of the catalytic cycle of this peroxidase were obtained by adding 1 (Compound I) or 2 or 8-10 (Compound II) mole-eq of H_2O_2 into the enzyme solution. Molar concentration of the enzyme was calculated assuming its molecular weight to be 43 kD [1]. The concentration of H_2O_2 was determined accord-

ing to the absorbance at 240 nm ($\varepsilon_{240} = 39.6\ mM^{-1}\cdot cm^{-1}$) [17].

Electrophoresis. SDS-PAGE was performed in 12% gel [18]. The protein was stained with Coomassie R-250.

Protein concentration was determined according to Lowry [19].

RESULTS

Submerged cultivation of *P. tigrinus*. During the cultivation of *P. tigrinus* on Kirk medium, which was limited by nitrogen and contained 0.32 mg/liter basic concentration of Mn^{2+} [15] without any inducers, trace activity of hybrid Mn-peroxidase and laccase was detected. However, the activity of the former did not exceed 0.02 U determined by NADH oxidation (data not shown). When the less sensitive technique was employed (Mn^{2+} oxidation to Mn^{3+}–malonate), the activity of hybrid Mn-peroxidase was not detected at all. When manganese concentration in the medium was increased to 71 mg/liter [16], the hybrid Mn-peroxidase activity reached 0.9 U (determined by Mn^{2+} oxidation), and laccase activity was also detected (Fig. 1). However, activity maxima of laccase (14-16th days) and hybrid Mn-peroxidase (19th-23rd days) did not overlap, which facilitated the further purification of the latter: at the peak of hybrid Mn-peroxidase activity the laccase activity was decreased 3-6 times compared to the maximum.

Purification of hybrid Mn-peroxidase. During the purification of this enzyme from the submerged *P. tigrinus*

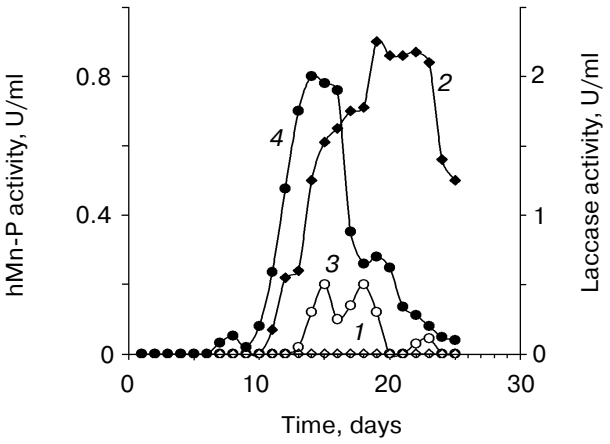

Fig. 1. Dynamics of hybrid Mn-peroxidase (hMn-P) (*1*, *2*) and laccase (*3*, *4*) activity in submerged culture of the fungus *P. tigrinus* 8/18 at Mn^{2+} concentration 0.32 (*1*, *3*) and 71 mg/liter (*2*, *4*).

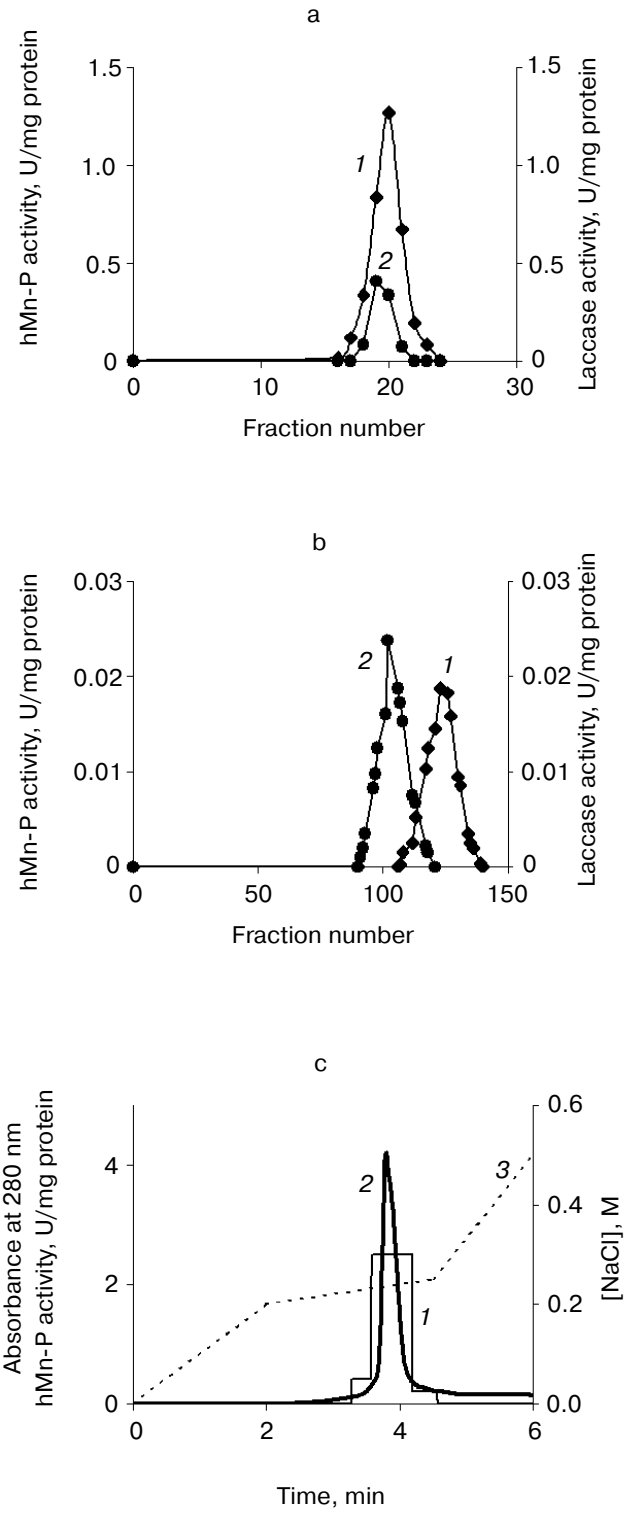

Fig. 2. Purification of hybrid Mn-peroxidase (hMn-P): a) elution profile of hMn-P (*1*) and laccase (*2*) after ion-exchange chromatography on TEAE-Servacell 23; b) gel filtration on Sephadex G-100; c) elution profile of hMn-P after ion-exchange chromatography on Mono-Q. *1*) Activity; *2*) absorbance at 280 nm; *3*) gradient of NaCl concentration (M).

Table 1. Purification of hybrid Mn-peroxidase from the fungus *P. tigrinus* 8/18

Purification stage	Volume, ml	Protein content, mg	Total activity, U	Specific activity × 10^3, U/mg protein	Purification degree
Culture broth	2930	26100	1143	0.044	1
Ion-exchange chromatography (TEAE-Servacell 23)	308	2580	737	0.29	6.6
Gel filtration (Sephadex G-100)	43	344	391	1.14	25.9
Ion-exchange chromatography (Mono Q HR 5/5)	2	45	112.5	2.5	56.8
Gel filtration (Sephacryl S-100, HiPrep 16/60)	4	3.1	11.4	3.7	84

culture, the main challenge was a separation of hybrid Mn-peroxidase from laccase. Separation from laccase was not achieved after chromatography on TEAE-Servacell (Fig. 2a). Subsequent gel filtration on Sephadex G-100 separated the enzymes, however, not completely (Fig. 2b). To avoid the contamination of hybrid Mn-peroxidase product by laccase, only those fractions were collected together, which did not contain the laccase activity. At the next stage (anion-exchange chromatography on Mono Q) the laccase activity was not detected; hybrid Mn-peroxidase was eluted at 0.23 M NaCl within a 0-0.5 M NaCl concentration gradient (Fig. 2c). At this and also the final step of enzyme purification (by gel filtration on Sephacryl S-100), the separation of hybrid Mn-peroxidase from minor protein contaminations was achieved. After gel filtration, the purified peroxidase was concentrated and transferred from buffer A with 0.1 M NaCl into bidistilled water. The results of purification are presented in Table 1.

The purified hybrid Mn-peroxidase was electrophoretically homogeneous (Fig. 3); the purity value RZ (A_{406}/A_{280}) was 4.3.

Substrate specificity of the hybrid Mn-peroxidase. The purified hybrid Mn-peroxidase catalyzed the typical reactions of the standard Mn-peroxidase: oxidase reaction (oxidation of NADH in the absence of H_2O_2) and peroxidase reaction. As a peroxidase, the hybrid Mn-peroxidase of *P. tigrinus* demonstrated H_2O_2-dependent oxidation of Mn^{2+} to Mn^{3+} with the formation of Mn^{3+}–malonate complexes, and also Mn^{2+}-dependent oxidation of "phenol oxidase" substrates (phenols: hydroquinone, guaiacol, 2,6-dimethoxyphenol, 2,4,6-trichlorophenol; aromatic amines: *p*-phenylenediamine, *o*-dianisidine; and ABTS).

However, in the absence of Mn^{2+} this enzyme catalyzed H_2O_2-dependent oxidation of ABTS, *p*-phenylenediamine, *o*-dianisidine, hydroquinone, and 2,6-dimethoxyphenol analogous to the classic horseradish peroxidase or lignin peroxidase (Table 2). The

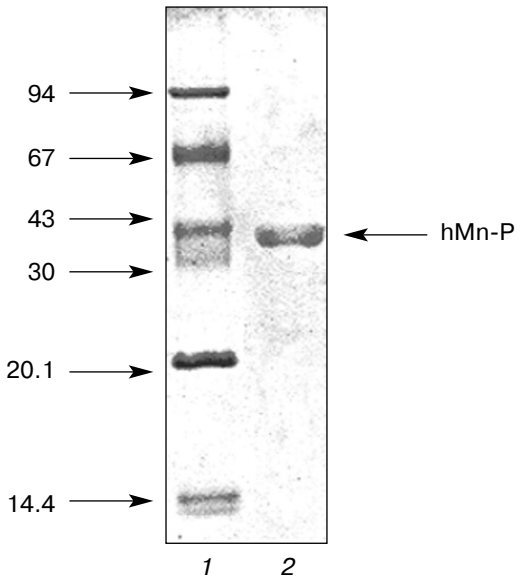

Fig. 3. SDS-PAGE of *P. tigrinus* 8/18 hybrid Mn-peroxidase (hMn-P, 10 μg) (*2*): *1*) marker proteins: phosphorylase B (94), bovine serum albumin (67), ovalbumin (43), carboanhydrase (30), soybean trypsin inhibitor (20.1), α-lactalbumin (14.4). To the left, molecular weights in kD.

Table 2. Substrate specificity of hybrid Mn-peroxidase from *P. tigrinus*

Substrate	λ_{max}, nm	Activity, U/mg protein	
		+ Mn	– Mn
ABTS	436	120	52
p-Phenylenediamine	320	210	20
o-Dianisidine	470	98	27
2,6-Dimethoxyphenol	469	148	1
Hydroquinone	254	358	58.8
Guaiacol	470	12.4	—
2,4,6-Trichlorophenol	255	38	—

oxidation rates for these substrates drastically increased in the presence of manganese. Guaiacol and 2,4,6-trichlorophenol were not oxidized without manganese.

Obtaining the catalytic cycle intermediates. The ability of hybrid Mn-peroxidase to oxidize certain substrates without Mn^{2+} suggested an unusual catalytic cycle for this enzyme, since the known fungal peroxidases oxidized the substrate either in the presence of manganese (Mn-peroxidase) or were independent of manganese (classic peroxidase like horseradish peroxidase or lignin peroxidase).

The absorption spectrum of the hybrid Mn-peroxidase was typical for a native form of a heme-containing peroxidase with intense absorbance in the Soret band at 406 nm and maxima in the visible range at 504 and 638 nm (Fig. 4, Table 3).

To obtain the two-electron oxidized intermediate of the peroxidase catalytic cycle (Compound I), 1 mole-eq of H_2O_2 was added to the native form of the hybrid Mn-peroxidase. In this case, the absorbance value in Soret band at 406 nm decreased to 40% of the native enzyme value, and maxima in the visible range were shifted to 528 and 642 nm (Fig. 5, Table 3). The resulting Compound I was unstable; it existed for no longer than 5 sec and after that spontaneously transformed into the native enzyme.

To obtain one-electron oxidized Compound II, 2-10 mole-eq of H_2O_2 were added to the native enzyme. The Soret band of Compound II shifted to 420 nm, and its absorbance was 60% of that for the native form. Absorption maxima in the visible range were found at 530 and 558 nm (Fig. 6, Table 3). Compound II was stable for no longer than 1 min when 2 mole-eq of H_2O_2 were added to the native peroxidase; and at least for 10 min after the addition of 8-10 mole-eq. In both cases, the subsequent spontaneous transformation of Compound II into the native enzyme consisted of two phases—fast and slow. During 30 min of the fast phase all spectral characteristics of the native enzyme were recovered except for the absorbance value in Soret band (which was 90-95% of native enzyme absorbance at 406 nm). During the slow phase the absorption intensity reached 100% within several hours. Five isobestic points (at 414, 461, 540, 624, and 660 nm) were observed for the reduction of Compound II into the native enzyme, which indicates a direct reaction without intermediates (Fig. 7).

Spectral characteristics of the catalytic cycle intermediates of hybrid Mn-peroxidase were similar to the properties of Compounds I and II of ligninolytic peroxidases from the fungus *P. chrysosporium* (Table 3).

Catalytic cycle of the hybrid Mn-peroxidase. Taking into account the different absorbance values in Soret bands for the redox cycle intermediates of hybrid Mn-peroxidase at 406 nm, transformations of the intermediates were observed according to the change of absorbance at this wavelength, analogous to [5].

Due to the instability of Compound I transformations native enzyme → Compound I and Compound I → Compound II could not be detected. A stable Compound II was obtained by adding 10 mole-eq of H_2O_2 to the native enzyme. When 1 mole-eq of Mn^{2+} or hydroquinone was added, Compound II reverted into the native enzyme within 2.5-3 min (Fig. 8, a and b). After the addition of 10 mole-eq of Mn^{2+} or hydroquinone this reaction

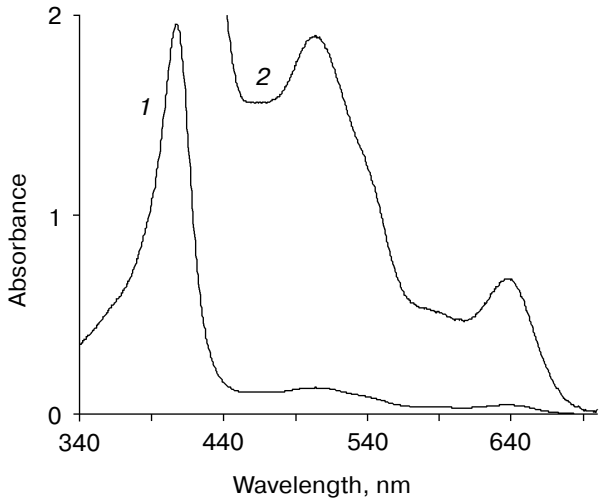

Fig. 4. Absorption spectrum of native hybrid Mn-peroxidase from *P. tigrinus* 8/18: *1*) absorption spectrum within the 340-700 nm range, maxima at 406, 504, and 538 nm; *2*) absorption maxima in visible range of the spectrum, 7 times magnification. Protein concentration in bidistilled water was 1 mg/ml.

Table 3. Spectral properties of hybrid Mn-peroxidase from *P. tigrinus* and other peroxidases

Enzyme	Native form	Compound I	Compound II	Reference
Hybrid Mn-peroxidase from *P. tigrinus*	406, 504, 638	406, 528, 642	420, 530, 558	this work
Mn-Peroxidase from *P. chrysosporium*	406, 502, 632	407, 558, 650	420, 528, 555	[7, 8]
Lignin peroxidase from *P. chrysosporium*	407, 500, 632	408, 550, 650	420, 525, 556	[7, 8, 20]
Horseradish peroxidase	403, 498, 640	400, 557, 650	420, 527, 554	[21]

took 10-30 sec (data not shown). In both cases a fast and a slow phase were observed.

DISCUSSION

Submerged cultivation of *P. tigrinus*. Typical white rot fungi initiate the production of ligninolytic enzymes during the transition to the secondary metabolism caused by nitrogen, sulfur, or carbon deficiency [15]. *P. tigrinus* 8/18 is a ligninolytic fungi species that is able to exhibit low ligninolytic activity also on rich media. However, the ligninolytic activity increases drastically at low concentrations of these elements. The need for high manganese concentration in the media is due to a special composition of extracellular ligninolytic enzymatic complex: *P. tigrinus* does not produce lignin peroxidase. For those fungi that are producing lignin peroxidase (e.g., *P. chrysosporium*) the increase in manganese concentration in the medium increases the yield of Mn-peroxidase, but also results in suppressed expression of lignin peroxidase and total ligninolytic activity of the culture [22, 23]. In the case of *P. tigrinus*, the increase in manganese concentration is also accompanied by increased yield of hybrid Mn-peroxidase, but the total ligninolytic activity of the culture correlates with the activity of this enzyme [16]. Increased manganese concentration induced laccase expression in the absence of special inducers in the

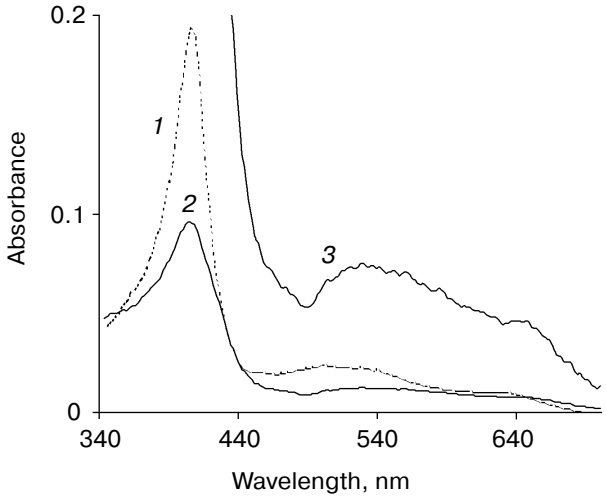

Fig. 5. Absorption spectrum of Compound I of *P. tigrinus* 8/18 hybrid Mn-peroxidase: *1*) absorption spectrum of the native form of the enzyme; *2*) absorption spectrum of Compound I in 340-700 nm range, maxima at 406, 528, and 642 nm; *3*) absorption maxima in the visible range of the spectrum, 5 times magnification. To obtain Compound I, 1 mole-eq of H_2O_2 was added to 3 nmol of native hybrid Mn-peroxidase in bidistilled water.

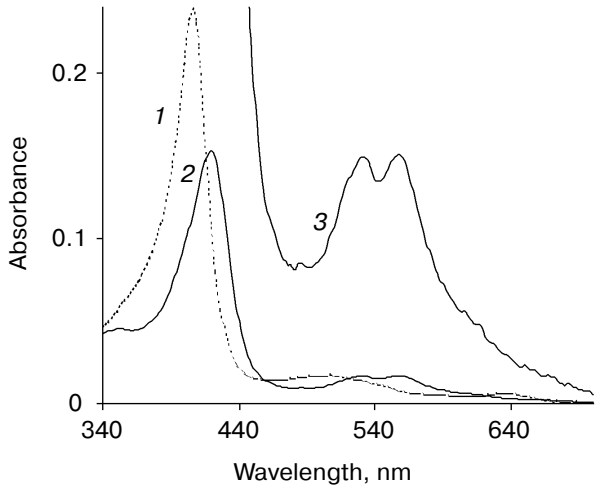

Fig. 6. Absorption spectrum of Compound II of *P. tigrinus* 8/18 hybrid Mn-peroxidase: *1*) absorption spectrum of the native form of the enzyme; *2*) absorption spectrum of Compound II in the 340-700 nm range, maxima at 420, 530, and 558 nm; *3*) absorption maxima in the visible spectrum range, 5 times magnification. To obtain Compound II, 10 mole-eq of H_2O_2 were added to 2 nmol of native hybrid Mn-peroxidase in bidistilled water.

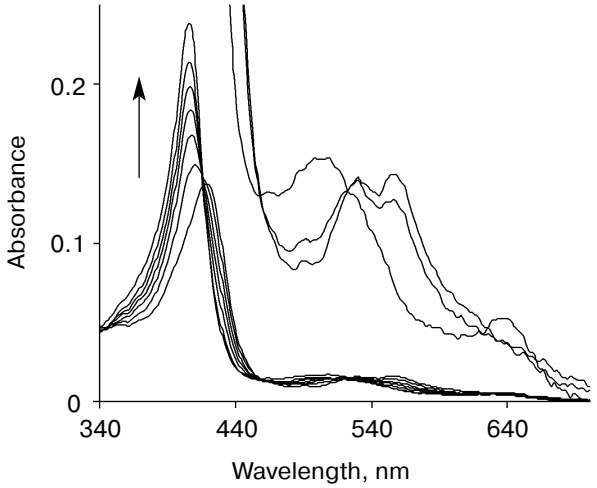

Fig. 7. Differential absorption spectrum of Compound II reduction into the native form of *P. tigrinus* 8/18 hybrid Mn-peroxidase. Spectra were recorded with 1.5 min interval. The arrow indicates the direction of the reaction. Inset: 5 times magnification.

medium (Fig. 1). The ability of manganese to induce laccase in certain ligninolytic fungi has been reported [24], but for *P. tigrinus* we described it for the first time. Different time of maximal laccase and hybrid Mn-peroxidase production enabled the further purification of peroxidase.

Purification of hybrid Mn-peroxidase. The most attention during the purification of the enzyme was paid to the separation from laccase and also to the high purity grade, sacrificing the yield. Even trace activity of laccase had to be avoided as it was necessary for the study of Mn-independent peroxidase activity, and high purity grade was needed to obtain stable intermediates of the catalytic cycle. As a result, the hybrid Mn-peroxidase was purified 84-fold with a high purity parameter RZ of 4.3; enzyme yield was 1-4%.

Substrate specificity. The ability of the hybrid Mn-peroxidase of *P. tigrinus* 8/18 to oxidize NADH as an oxidase and also oxidize a number of characteristic substrates in the presence of Mn^{2+} and H_2O_2, especially oxidation of $MnSO_4$ forming Mn^{3+}–malonate complex, is a usual property of a typical Mn-peroxidase from ligninolytic fungi. However, the detection of peroxidase reactions of this enzyme with the number of substrates in the absence of manganese indicates its affiliation to the group of hybrid Mn-peroxidases. The usual Mn-peroxidase is not able to oxidize the substrates without manganese [7].

Besides the hybrid Mn-peroxidase of *P. tigrinus* 8/18 isolated by us from solid-state culture [8, 9], similar enzymes were found in *Bjerkandera* sp. BOS55 [11], *Bjerkandera adusta* [12, 14], *Pleurotus eryngii* [13], and *Pleurotus pulmonarius* [25]. Probably, the enzyme from *Ceriporiopsis subvermispora*, classified by the authors as a Mn-peroxidase was in fact a hybrid Mn-peroxidase because *p*-anisidine and *o*-diansidine were oxidized by this peroxidase in the absence of manganese ions [26]. The substrate specificity of hybrid peroxidases varies between different fungi: unlike the hybrid Mn-peroxidase from *P. tigrinus* 8/18 and *Bjerkandera adusta* 90-41, the enzyme from *Bjerkandera* sp. BOS55 oxidized guaiacol and 2,6-dimethoxyphenol in the absence of Mn^{2+}.

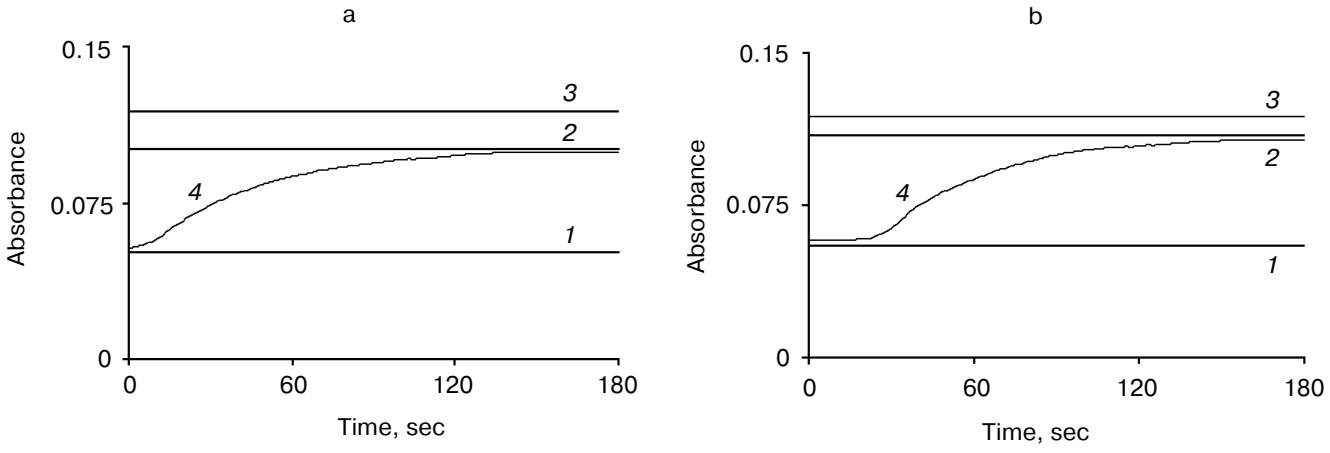

Fig. 8. Absorption change at 406 nm during the reduction of Compound II into the native form of *P. tigrinus* 8/18 hybrid Mn-peroxidase: *1*) absorption of Compound II at 406 nm; *2*) absorption of native enzyme at 406 nm after the fast phase of the reaction of Compound II; *3*) absorption of native enzyme at 406 nm after the slow phase of the reaction of Compound II; *4*) absorption change at 406 nm during the fast phase of reaction of Compound II into the native enzyme. Reduction of 1 nmol of Compound II by adding 1 mole-eq of $MnSO_4$ (a) or hydroquinone (b).

Catalytic cycle intermediates. Spectral characteristics of the catalytic cycle intermediates of *P. tigrinus* hybrid Mn-peroxidase were similar to those of ligninolytic peroxidases (lignin- and Mn-peroxidases) from *P. chrysosporium*, and different from the properties of horseradish peroxidase (Table 3). The formation of Compound II not only resulted in the shift of Soret band toward the red region of the spectrum, but also in a decrease of its absorption intensity by 40% compared to the native enzyme. This decrease in absorption intensity was also observed for ligninolytic lignin- and Mn-peroxidases from *P. chrysosporium* [5, 27]. Such decrease in absorption intensity was not observed for horseradish peroxidase [21] and non-ligninolytic peroxidase from *Coprinus macrorhizus* [28], thus being an additional indication about the affiliation of *P. tigrinus* hybrid Mn-peroxidase to ligninolytic peroxidases.

Catalytic cycle. A general scheme of catalytic cycle for heme-containing peroxidases is the same for horseradish peroxidase [21] and ligninolytic lignin- and Mn-peroxidases from the Basidiomycete *P. chrysosporium* [5, 27]:

$$\text{peroxidase} + H_2O_2 \rightarrow \text{Compound I} + H_2O,$$
$$\text{Compound I} + AH_2 \rightarrow \text{Compound II} + AH^{\cdot},$$
$$\text{Compound II} + AH_2 \rightarrow \text{peroxidase} + AH^{\cdot},$$

where Compounds I and II are redox cycle intermediates, AH_2 the reducing substrate, and $AH^{\cdot}$ the radical product of the reducing substrate oxidation.

According to this scheme, the result of an interaction between the native form of peroxidase and H_2O_2 is a formation of two-electron oxidized Compound I, which transforms back to the native enzyme after two subsequent acts of one-electron reduction by the reducing substrate. In the catalytic cycle of lignin peroxidase the role of the reducing substrate is played by organic compounds, for instance lignin or fragments of its degradation [27]. Mn^{2+} is not a lignin peroxidase substrate, and thus cannot reduce redox cycle intermediates of this enzyme [29, 30]. In the catalytic cycle of Mn-peroxidase Compound I can be reduced to Compound II either by an organic substrate (preferably of phenolic nature) or by Mn^{2+}. However, in the last reaction of the Mn-peroxidase cycle Compound II is reduced to the native form only by manganese [5]. Hence, the principal difference between the catalytic cycles of lignin and Mn-peroxidases is the final reaction of reduction of Compound II.

The presented data indicate that the reduction of Compound II of *P. tigrinus* hybrid Mn-peroxidase was mediated by Mn^{2+} as well as by an organic compound, hydroquinone. This means that the catalytic cycle of *P. tigrinus* hybrid Mn-peroxidase has similarities with those of lignin- and Mn-peroxidases. As presented by us earlier, *P. tigrinus* hybrid Mn-peroxidase extracted from the solid-state culture has the same unusual redox cycle [8]. Analogous organization of the redox cycle was also reported for hybrid Mn-peroxidase of *Pleurotus eryngii* [13].

The detection of hybrid Mn-peroxidase in both solid-state and submerged cultures of *P. tigrinus*, *P. eryngii*, and other similar fungi indicates that the production of these enzymes does not depend on the cultivation technique, and is an essential condition for functioning of the ligninolytic system as a whole.

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