ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 9, pp. 1027-1034. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © Y. G. Pakhadnia, N. I. Malinouski, A. G. Lapko, 2009, published in Biokhimiya, 2009, Vol. 74, No. 9, pp. 1260-1269.

Purification and Characteristics of an Enzyme with Both Bilirubin Oxidase and Laccase Activities from Mycelium of the Basidiomycete *Pleurotus ostreatus*

Y. G. Pakhadnia[1]*, N. I. Malinouski[2], and A. G. Lapko[1]

[1]*Sakharov International Environmental University, Dolgobrodskaya ul. 23, 220009 Minsk, Belarus; fax: (017) 230-6897; E-mail: pohodnia@yahoo.com; alapko2002@yahoo.com*
[2]*Department of Biochemistry, University of Nebraska, Lincoln 68588-0664, USA; E-mail: mmalinouski@genomics.unl.edu*

Received August 26, 2008
Revision received October 16, 2008

Abstract—A homogenous enzyme with both bilirubin oxidase and laccase activities was isolated from a submerged culture of the basidiomycete *Pleurotus ostreatus* mycelium and characterized. The yield of the enzyme was 127 μg/g dry biomass of the mycelium. The specific activity of the enzyme was 21 and 261 U/mg to bilirubin and to a laccase substrate ABTS, respectively. The intracellular phenol oxidase from the *P. ostreatus* mycelium was identified as bilirubin oxidase with the amino acid sequence highly homologous to that of the *pox2* gene-encoded product. The enzyme displayed the maximal laccase activity at 50-55°C to all substrates examined, whereas the pH optimum was substrate-dependent and changed from 3.0 for ABTS to 7.0 for syringaldazine and guaiacol. The enzyme maintained catalytic activity within a broad pH range but was inactivated at pH 4.0. The enzyme was thermostable but very sensitive to metal chelating inhibitors. Trypan Blue (5 mg/liter) was completely decolorizated upon 3 h of incubation with the bilirubin oxidase (20 mU/ml) at room temperature.

DOI: 10.1134/S0006297909090119

Key words: mycelium, *Pleurotus ostreatus*, oxidoreductase, bilirubin oxidase, laccase

Basidiomycetes belong to a family of white rot fungi known for being good producers of laccases and bilirubin oxidases. The active sites of these oxidoreductases contain metals (copper, zinc, iron) liganded by histidine and cysteine residues [1, 2]. Laccases (*p*-diphenol:oxygen oxidoreductases, EC 1.10.3.2) are the most important enzymes of the ligninolytic complex of wood-destroying white rot fungi [3]. The natural function of the enzyme determines its extracellular localization. Features of extracellular laccases of Basidiomycetes are studied in many works [4]. Basidiomycetes can also produce intracellular laccases [5]. The functions of intracellular laccases are now intensively discussed.

Bilirubin oxidase (BOX, EC 1.3.3.5) catalyzes bilirubin transformation to biliverdin. Basidial fungi are often used as commercial producers of bilirubin oxidases [6-8]. The laccase activity has been recently detected in bilirubin oxidases isolated from the culture medium of Basidiomycetes [9], whereas laccases cannot convert bilirubin to biliverdin [10-12]. The primary structure of enzymes capable of oxidizing bilirubin and transforming laccase substrates is usually very similar to that of laccases, and such enzymes are called bilirubin oxidases/laccases. In particular, such an enzyme is exemplified by BOX from the basidiomycete *Pleurotus ostreatus*, which was prepared as a recombinant protein by cloning the corresponding cDNA [13]. The purified recombinant protein displayed both laccase and bilirubin oxidase activities, and the primary structure of BOX had 97% identity to the structure of the *pox2* gene product defined as a corresponding laccase. Nevertheless, BOX could not be isolated from its natural source, the fungus *P. ostreatus*, because the enzyme was poorly accumulated in the culture medium (1 U/ml) [13], which prevented its purification and characterization.

In our study we found that BOX from *P. ostreatus* was an intracellular protein and that its production increased with accumulation of the fungal mycelium biomass. The enzyme was isolated, purified to homogeneity, and its

Abbreviations: ABTS, 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); BOX, bilirubin oxidase; DMP, 2,6-dimethoxyphenol; DTT, dithiothreitol; SGZ, syringaldazine; TB, Trypan Blue.

* To whom correspondence should be addressed.

structure and physicochemical properties were determined. The intracellular BOX capable of oxidizing bilirubin can also transform a variety of laccase substrates, including dyes, and rates of these reactions are similar to activities of extracellular laccases isolated from the cultural medium of *P. ostreatus* [14].

MATERIALS AND METHODS

Organism and growth conditions. The white rot fungus *P. ostreatus* 43 ((Fr.) P. Kummer) BIM F-306D was taken from the Collection of the Institute of Microbiology, National Academy of Sciences of Belarus. The fungal mycelium was grown on brewer's wort (8° by Balling) with initial pH value of 6.0. The fungus was grown for 10 days on a circular shaker at 180 rpm and 25-26°C. From the second day of the mycelium growth, every 24 h the parameters were determined as follows: biomass, protein concentration in the mycelium, activities of intra- and extracellular laccases to the ABTS substrate (2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid), and also the activity of the intracellular bilirubin oxidase. The yield of biomass was determined by weighing the mycelium previously washed and filtered across a dense synthetic tissue, and the absolute dry mass of the mycelium was calculated considering the humidity coefficient.

Isolation and purification of the enzyme. To characterize the intracellular oxidase activity, on the seventh day of growth the mycelium biomass was separated from the nutrient medium by centrifugation at 6000*g* for 10 min and washed many times with 10 mM Tris/sodium citrate buffer (pH 7.4) to virtually completely remove the extracellular laccase activity.

The *P. ostreatus* mycelium was suspended in 50 mM Tris/sodium citrate buffer (pH 7.4) (buffer A), homogenized for 10 min at 22,000 rpm using a Waring Commercial blender (USA), and frozen at −20°C. The mycelial homogenate was defrosted rapidly, and the cells were disintegrated by ultrasound with a UZDN-2T instrument (Russia) at 22 kHz for 5 min on ice. The supernatant resulting by centrifugation at 30,000*g* for 30 min was separated and used for isolating the protein.

The supernatant was placed onto a column (2 × 10 cm) with DEAE-cellulose equilibrated with buffer A. The adsorbed matter was eluted with a linear gradient of ammonium sulfate (from 0 to 0.7 M) in the same buffer. Fractions with oxidoreductase activity from the ion-exchange chromatography were separated on a column (1 × 5 cm) with ConA-Sepharose equilibrated with buffer A. Nonspecifically adsorbed material was washed out with 0.7 M $(NH_4)_2SO_4$, and the protein bound with the affinity matrix was eluted with 0.5 M solution of methyl-β-D-glucopyranoside in buffer A. The fraction with oxidoreductase activity was subjected to gel chromatography on a column (1.5 × 70 cm) with Toyopearl HW-55 equilibrated with buffer A supplemented with 0.3 M $(NH_4)_2SO_4$ under conditions of isocratic elution. The active fraction of the enzyme was collected and dialyzed against 10 mM buffer A (pH 7.4). After dialysis, the enzyme solution was stored in 20% glycerol at −20°C.

Protein concentration was determined by Bradford's method [15].

Determination of molecular mass. SDS-PAGE was performed in 12% polyacrylamide gel by the Laemmli method [16] with subsequent staining by Coomassie R-250 [17]. Phosphorylase B (96 kDa), BSA (67 kDa), aldolase (49 kDa), triosephosphate isomerase (27 kDa), and lysozyme (14.4 kDa) were used as marker proteins.

The molecular mass of the native oxidase was determined by FPLC gel filtration on a column with Toyopearl HW-55 (1.5 × 70 cm) equilibrated with buffer A supplemented with 0.3 M $(NH_4)_2SO_4$. The column was calibrated with BSA (67 kDa), protein A (41 kDa), and chymotrypsin (25 kDa) as standards.

Native electrophoresis of active fractions was performed in 12% polyacrylamide gel. Oxidoreductases were visualized by their enzymatic activities with a mixture for analyzing laccase activity. Stained bands were cut out and analyzed by mass spectrometry.

Determination of enzymatic activities. The laccase activity of the enzyme was determined by spectrophotometry [14] on addition of 10 µl of the enzyme solution to 1 ml of 1 mM ABTS (ε_{420} = 36,000 $M^{-1}\cdot cm^{-1}$). The substrate was dissolved in McIlvaine buffer (0.1 M citric acid/0.2 M NaH_2PO_4, pH 3.5) [18]. The enzyme amount converting 1 µmol of substrate in 1 min was taken as the unit (U) of the enzyme activity. Spectrophotometry was performed using a UV-2501 PC spectrophotometer (Shimadzu, Germany) equipped with a thermostatic chamber.

The bilirubin oxidase activity was determined using 0.002% bilirubin solution in McIlvaine buffer (pH 7.4). Bilirubin was oxidized to biliverdin at 37°C in a mixture of 0.99 ml of the bilirubin solution and 0.01 ml of the enzyme (45 mU). The oxidation rate was calculated from the decrease in the substrate absorption at 440 nm using molar absorption coefficient 56,300 $M^{-1}\cdot cm^{-1}$ [19].

Kinetic measurements. Michaelis constants of the purified enzyme were determined by spectrophotometry for the following substrates: ABTS (ε_{420} = 36,000 $M^{-1}\cdot cm^{-1}$), guaiacol (ε_{465} = 12,100 $M^{-1}\cdot cm^{-1}$), 2,6-dimethoxyphenol (DMP) (ε_{477} = 14,800 $M^{-1}\cdot cm^{-1}$), syringaldazine (SGZ) (ε_{525} = 64,000 $M^{-1}\cdot cm^{-1}$) [20] by absorption of the oxidation product at 25°C. The K_m value was calculated using the SigmaPlot program Enzyme Kinetics Modulus (USA).

To assess the influence of pH on the rate of the enzymatic reactions, the laccase activity was determined with ABTS, guaiacol, DMP, and SGZ at pH 2.2-8.0 in McIlvaine universal buffer system with the step of 0.5 pH unit.

To study the stability of the oxidase at pH 2.2-9.0, the enzyme solutions in McIlvaine buffers were incubated at 25°C. After 30, 60, 120, and 180 min of incubation, enzyme aliquots (10 μl) were taken and added to 1 ml of 1 mM solution of ABTS in McIlvaine buffer (pH 3.5), and the activity was measured as described above. The oxidase activity before the incubation was taken as 100%.

The influence of temperature on the enzymatic reaction rate was assessed by the ability of the enzyme to oxidize ABTS at temperatures in the 20-75°C range.

The thermostability of the enzyme was determined by changes in the laccase activity after incubation of the oxidoreductase for 3 h in McIlvaine buffer (pH 7.4) at 40, 50, and 60°C. The enzyme activity was measured with ABTS as a substrate as described above. The oxidase activity at 25°C before the incubation was taken as 100%.

The effect of inhibitors on the enzyme activity was studied at 25°C with 1 mM ABTS as a substrate.

Decolorization of dye. The rate of decolorization of the azo dye Trypan Blue (TB) in the presence of the purified enzyme preparation was determined spectrophotometrically by changes in the dye absorption at 583 nm. The reaction mixture consisted of TB (5 mg/liter) dissolved in McIlvaine buffer (pH 3.5) and laccase (20 mU/ml). The TB decolorization was determined every 20 min during the first hour and every 60 min during the subsequent 2 h.

Mass-spectrometry (ESI-MS/MS) was performed using a Q-TOF Ultima mass spectrometer (Micromass, Great Britain). The protein material of the corresponding bands of the native electrophoretic gel was alkylated with iodoacetamide and salted out on a Vydac C4 pre-column and then treated with trypsin in 50 mM Tris-HCl buffer (pH 8.2) for 8 h at 37°C. The tryptic peptides were analyzed by capillary reversed-phase chromatography and ESI-MS/MS. The mass spectrometer was used in scanning regime of 200-2000 m/z. To identify the peptide structure, the three most intense ions of each scan were subjected to MS/MS.

Proteins were identified with Mascot programs comparing the results with the UniProtKB international database of tryptic hydrolyzates.

Reagents used were as follows: DEAE-cellulose (Serva, USA); ConA-Sepharose, a set of marker proteins for gel chromatography and electrophoresis, EDTA, methyl-β-D-glucopyranoside, ABTS, guaiacol, DMP, SGZ, TB (Sigma, USA); Toyopearl HW-55 (Tosoh, Japan); Coomassie Blue R-250, Tris(hydroxymethyl)aminomethane, glycine, acrylamide, N,N′-methylenebisacrylamide (Serva, Germany); 2-mercaptoethanol (Ferak, Germany); bilirubin (Fluka, Germany); Na_2HPO_4, peptone (Merck, Germany); glycerol, ethanol, CH_3COOH, H_3PO_4, $(NH_4)_2SO_4$, KH_2PO_4, K_2HPO_4, glucose, $MgSO_4 \cdot 7H_2O$, urea, dithiothreitol (DTT), sodium citrate (preparations of special and chemical purity) (Analyz X, Belarus). All solutions were prepared with water purified on a Falk apparatus (Falk, Germany).

All experiments were repeated five times.

RESULTS

Organism and growth conditions. Time-dependent changes in the laccase activity during mycelium growth. The white rot fungus *P. ostreatus* is a good producer of highly active extracellular laccases [4, 14, 21] catalyzing oxidation of *ortho*- and *para*-diphenols, aminophenols, polyphenols, polyamines, lignin phenolic substructures, and also some inorganic ions with an associated reduction of molecular oxygen to water. Information about intracellular isoforms of this enzyme is fragmentary.

Initially, we studied time-dependent changes in the specific enzymatic activity of the intracellular and extracellular oxidoreductases and in the increase in the basidiomycete mycelium biomass. Samples for analysis of these parameters were taken every day for 10 days. The maximum specific activities of both oxidoreductases were recorded on the fourth day of the mycelium growth and equaled 24 U/mg for extra- and intracellular enzyme (Fig. 1a).

Notwithstanding the progress in accumulation of the mycelium biomass, the oxidase activities of both enzyme isoforms noticeably decreased on the 5th-7th days of the growth and continued to lower. Thus, by the 10th day of the cultivation specific activities of the enzyme were 53 and 76% of the maximal values of the extra- and intracellular oxidoreductases, respectively.

To characterize the intracellular oxidase activity, the mycelium was collected on the 7th day of growth and thoroughly washed with buffer to eliminate the extracellular laccase activity. Mass of mycelium wrung out on a filter from 1 liter of culture medium was 49.2 g at humidity of ~88%. The mycelium was disintegrated by twofold freezing, thawing, and ultrasonication. The supernatant resulting from centrifugation of the *P. ostreatus* mycelium homogenate had yellow-brown color and was used for isolating the intracellular oxidase.

Purification of virtually of all known laccases includes a traditional stage of anion-exchange chromatography [13, 14, 22]; therefore, DEAE-cellulose was used to concentrate and crudely purify the enzyme. The oxidase activity peak was eluted from the ion-exchange sorbent with 0.1 M $(NH_4)_2SO_4$. The resulting enzyme preparation was light yellow and in addition to the pigment component also contained admixtures of other proteins.

The majority of known laccases are glycoproteins [3] containing 10-20% carbohydrates. Therefore, the next stage of the enzyme purification was affinity chromatography on a column with ConA-Sepharose. Nonspecifically adsorbed matter was washed out with 0.7 M $(NH_4)_2SO_4$, and the protein bound with the affinity matrix was eluted

with 0.5 M solution of methyl-β-D-glucopyranoside. A protein with oxidase activity was eluted as a single peak. Affinity chromatography as a stage of purification allowed us not only to nearly completely remove an accompanying protein component but also to separate the oxidoreductase from the pigment and increase eightfold the phenol oxidase activity of the sample (Table 1).

The molecular mass of the intracellular oxidoreductase from *P. ostreatus* determined by SDS-PAGE in 12% polyacrylamide gel was ~62 kDa (Fig. 2a), which corresponds to the average molecular mass of the majority of extracellular laccases [4].

The additional purification by gel-filtration chromatography resulted in an electrophoretically homogenous oxidoreductase preparation as a 60-kDa monomeric protein (data not presented). Results of electrophoresis in polyacrylamide gel under native conditions with subsequent detection of oxidoreductase by its ability to transform ABTS to a stained product confirmed the homogeneity of the preparation (Fig. 2b).

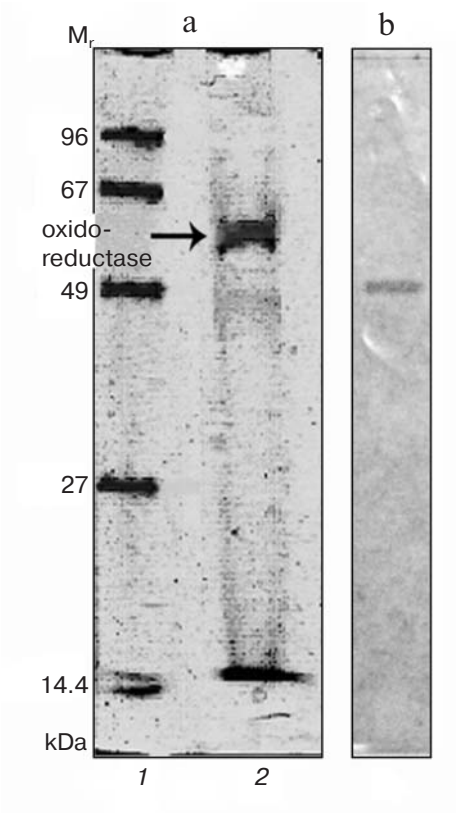

Fig. 2. Electrophoregrams in 12% polyacrylamide gel of purified intracellular oxidoreductase from *P. ostreatus* mycelium. a) Electrophoresis under denaturing conditions: *1*) marker proteins; *2*) a laccase specimen after affinity chromatography on ConA-Sepharose. b) Electrophoresis of the protein under native conditions with subsequent detection of oxidoreductase by the product of ABTS and SGZ transformation.

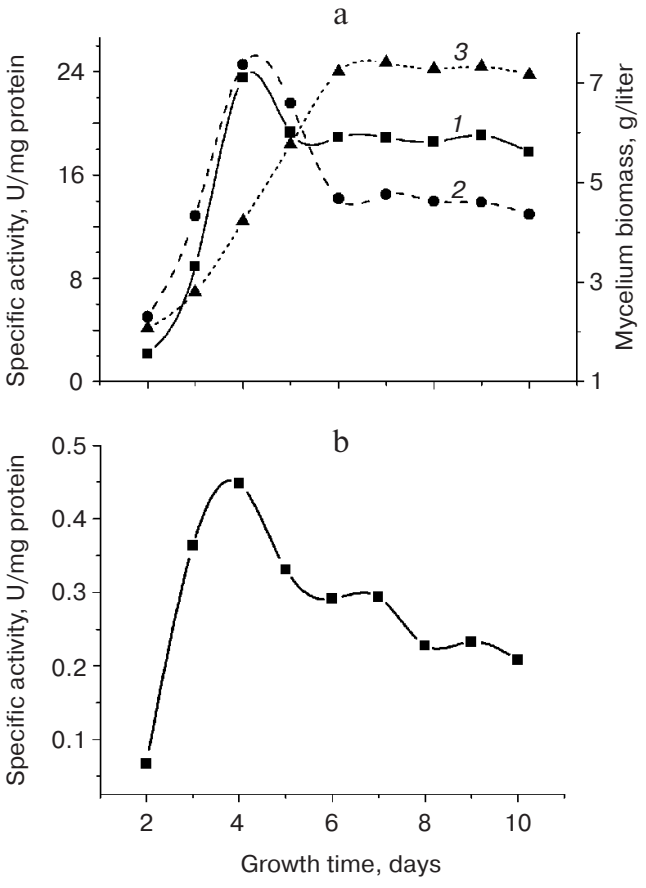

Fig. 1. a) Specific activities of intracellular (*1*) and extracellular (*2*) oxidoreductases and increase of the mycelium biomass (*3*) in dependence on the time course of the culture; b) changes in the specific activity of intracellular BOX from *P. ostreatus* during the time course of the culture.

Thus, the described succession of isolation and purification stages of the intracellular oxidoreductase allowed us to isolate from 5.9 g dry mycelium of *P. ostreatus* 0.75 mg protein with oxidoreductase activity which was 11.6% of the total activity of the mycelium homogenate. The specific activity of the purified enzyme preparation determined with ABTS was 261 U/mg.

The absorption spectrum of the studied protein did not have in the region of 220-800 nm a maximum at 610 nm specific for fungal laccases and corresponding to a type I copper atom. Earlier the absence of such a peak in the absorption spectrum was reported for yellow laccases of the fungi *P. ostreatus*, *Panus tigrinus*, and *Phlebia radiata* [21].

Mass spectrometry of the electrophoretically purified intracellular oxidoreductase allowed us to determine the amino acid sequence of five peptides resulting from tryptic hydrolysis of the protein:

^{52}SVPATDPTPATVSIPGVLVQGNK^{74},

^{75}GDNFQLNVVNQLSDTTMLK^{93},

Table 1. Purification of intracellular oxidoreductase from *P. ostreatus* mycelium

Purification step	Total protein, mg	Specific activity, U/mg	Recovery, %
Homogenate	88.5	19.1	100
DEAE-cellulose	8.25	126	62
ConA-Sepharose	1.61	158	15
Gel filtration	0.75	261	11.6

^{210}YAGGPTSPLSIINVESNKR^{228},

^{277}YSFVLTAD(N)^{284}QT(A)^{286}VGNYWIR^{293},

^{294}ANPNLGSTGFD(V)^{304}GGINSAILR^{313}.

Sequences of three peptides which envelop the sequence from the 52nd to 93rd amino acids from the *N*-end of the protein fully correspond to the primary structure of the product encoded by the *pox2* gene of the *P. ostreatus* genome. Two other peptides presented sequences from the 277th to the 313th residue. Three characteristic replacements are located in this region (D by N; T by A, and D by V in positions 284, 286, and 304, respectively), which indicated that the isolated enzyme is bilirubin oxidase (BOX; EC 1.3.3.5). The primary structure of BOX from *P. ostreatus* was established by the gene in 1999 [13]. According to the UniProtKB database (version of 05.02.2008), the bilirubin oxidase/laccase code is Q9UVY4_PLEOS, and this enzyme is a protein for which only transcriptional data are found, i.e. the existence of the transcript has been shown but the protein has not been characterized. Thus, the enzyme from the *P. ostreatus* mycelium, which was presumably characterized as an intracellular laccase, should be able to transform bilirubin to biliverdin. The oxidative ability of purified BOX assessed by Michaelis constant in the reaction with bilirubin was 55 μM, and the specific activity of the enzyme was 21 U/mg protein, which is within the range of parameters for bilirubin oxidases isolated from other Basidiomycetes [23].

The specific activity of the intracellular BOX in bilirubin oxidation to biliverdin was the highest on the fourth day of cultivation, which correlated with the total phenol oxidase activity measured with ABTS (Fig. 1b).

Primary structures of the intracellular BOX from *P. ostreatus* mycelium and of the gene *pox2* product had 97% identity [13]. Such a high identity of the proteins seems to explain the laccase activity of the isolated bilirubin oxidase. The laccase activity of BOX was studied with four substrates: three monophenols (DMP, guaiacol, and SGZ) and a diphenol (ABTS). The specific activity of the enzyme to these substrates decreased in the following series: ABTS (261 U/mg) > DMP (172 U/mg) > SGZ (76 U/mg) > guaiacol (25 U/mg); this correlated with the series for oxidation of substrates by the extracellular laccases from *P. ostreatus*.

Values of K_m for all laccase substrates obtained for BOX from *P. ostreatus* were lower than the corresponding values determined for other laccases isolated from the same source (Table 2). Values of K_m obtained by us for BOX are not unique – laccases from other Basidiomycetes have similar values. Thus, the laccase from *Trametes pubescens* is characterized by K_m = 14 μM for ABTS, K_m of laccase from *Thelephora terrestris* is 3 μM for

Table 2. Biochemical parameters of BOX and extracellular laccases from *P. ostreatus*

Enzyme	M_r, kDa	pH optimum				K_m, μM				Temperature optimum, °C	Inactivation half-period, h*
		ABTS	DMP	guaiacol	SGZ	ABTS	DMP	guaiacol	SGZ		
POXA1b	62	3.0	4.0-5.0	n.d.	6.0	370	260	n.d.	220	20-50	3 (60)
POXA1w	61	3.0	3.0-5.0	n.d.	6.0	90	2100	n.d.	130	45-65	3.3 (60)
POXA2	67	3.0	6.5	6.0	6.0	120	740	3100	140	25-35	0.2 (60)
POXA3a	83-85	3.6	5.5	6.2	n.d.	70	14 000	n.d.	36	35	6 (40)
POXA3b	83-85	3.6	5.5	6.2	n.d.	74	8800	n.d.	79	35	14 (40)
POXC	59	3.0	3.0-5.0	6.0	6.0	280	230	1200	20	50-60	0.5 (40)
BOX	62	3.5	6.0	7.0	7.0	14	16	45	3	50-55	0.3 (60)

Note: n.d., no data.

* Temperature (°C) is given in parentheses.

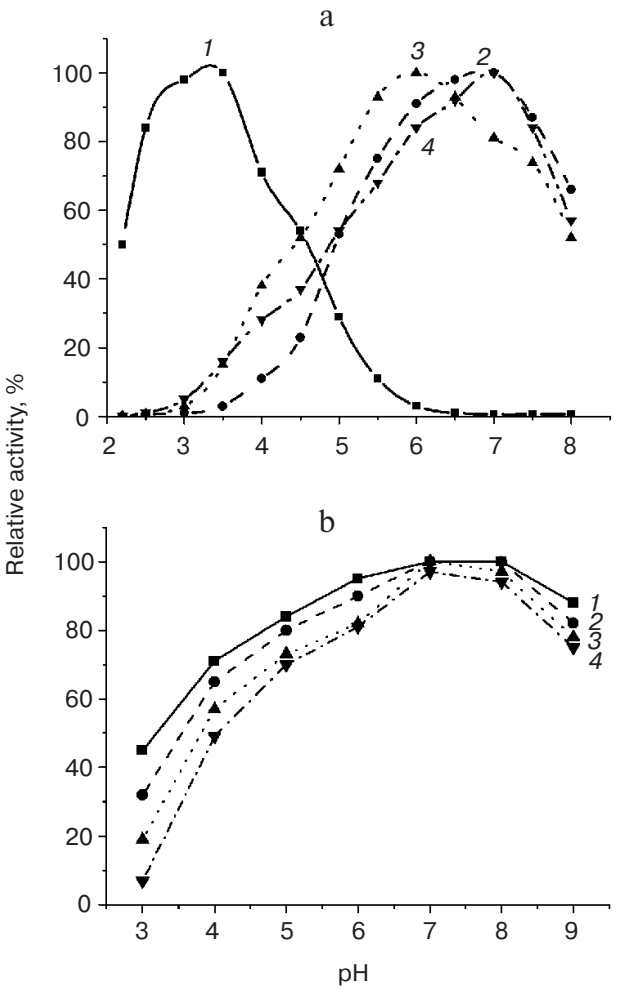

Fig. 3. a) pH profiles of oxidoreductase from *P. ostreatus* with ABTS, SGZ, DMP, and guaiacol as substrates (*1-4*, respectively); b) pH profiles during incubation for 30, 60, 120, and 240 min (*1-4*, respectively). All experiments were performed at 25°C in McIlvaine buffer.

SGZ, and K_m values calculated, respectively, for laccases from *T. versicolor* and *T. hirsute* are 15 μM for DMP and 63 μM for guaiacol [24-28].

The pH dependence of the activity of BOX from *P. ostreatus* was determined with the four above-mentioned substrates in the pH range from 2.2 to 8.0. The optimum catalytic activities of the enzyme were recorded at pH 3.5 with ABTS, 7.0 with SGZ and guaiacol, and 6.0 with DMP (Fig. 3a). Note that the activity levels at the extreme values of pH depended on the substrate. Thus, at pH 2.2, the enzyme activity with ABTS was 60% of the maximum, but no activity was recorded with the other substrates. On the contrary, at pH 8.0 the laccase activity with SGZ, DMP, and guaiacol was 57-66% of the maximum, whereas the activity with ABTS was virtually absent.

The pH range of the retained enzyme activity is an important characteristic for practice. Thus, the enzyme maintained 70% of the laccase activity upon incubation at 25°C for 4 h at pH 5.0-9.0 and was rather stable at weakly pH alkaline values (Fig. 3b). But at acidic pH the enzyme rapidly denatured, and after 4 h of incubation at pH 4.0 the activity of the BOX was not higher than 50% and at pH 3.0 it was less than 10% of the initial activity.

Note that changes in the oxidoreductase activity on heating and the stability of BOX at high temperature were similar to changes in these parameters recorded for other laccases from *P. ostreatus* (Table 2). Thus, the BOX activity increased with heating from 20 to 50°C up to maximum at 50-55°C and then sharply decreased at temperatures above 60°C (Fig. 4a). The enzyme was relatively thermostable at 40°C, and after incubation for 17 h its activity decreased by only 30%, whereas bilirubin oxidase from *T. tsunodae* lost 40% of its activity even after 30 min under the same conditions [29]. However, at 60°C BOX was half-inactivated even after 18 min (Fig. 4b).

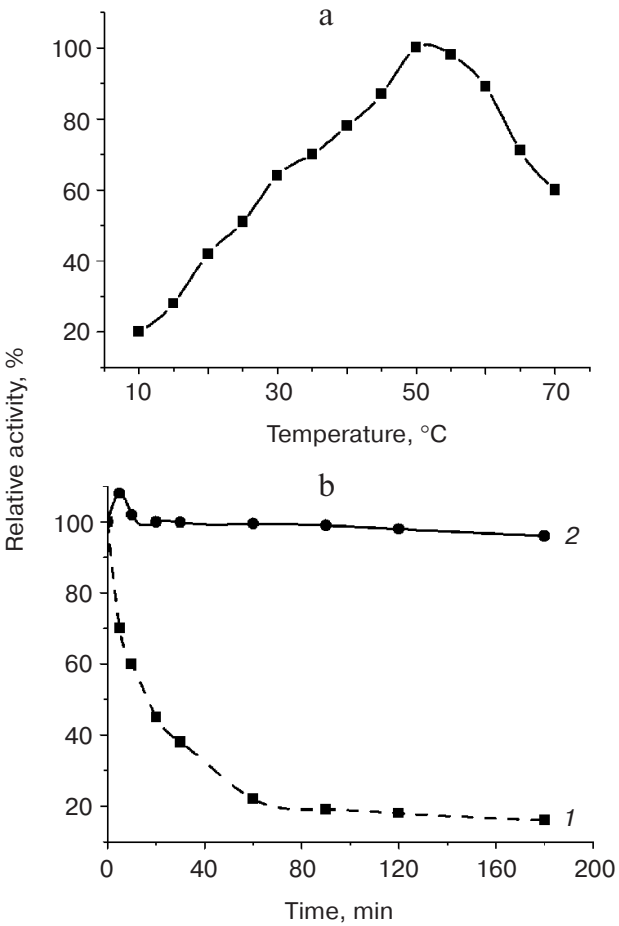

Fig. 4. Influence of temperature on the ability of BOX from *P. ostreatus* to transform ABTS in McIlvaine buffer (pH 3.5). a) Changes in the enzyme activity with increase in temperature from 10 to 70°C; b) BOX activity upon incubation in McIlvaine buffer (pH 7.4) for 180 min: *1*) at 60°C; *2*) at 40°C.

Table 3. Effects of inhibitors on activity of BOX from *P. ostreatus*

Inhibitor	Inhibitor concentration, mM	Relative activity, %
EDTA	0	100
	0.1	94 ± 1
	1	79 ± 1
	10	61 ± 2
	100	16 ± 2
Urea	0	100
	1000	85 ± 1
	2000	78 ± 2
	4000	72 ± 2
	5000	66 ± 2
DTT	0	100
	0.001	79 ± 1
	0.005	64 ± 1
	0.01	54 ± 1
	0.05	23 ± 1

The influence of inhibitors EDTA, DTT, and urea on the enzyme activity was studied (Table 3). EDTA is a weak inhibitor of fungal laccases because it removes bivalent metal ions from the active site of phenol oxidases. Increasing the EDTA concentration in the reaction mixture from 0.1 to 100 mM resulted in a decrease in the enzyme activity to 16% of the maximum, and this confirmed that the isolated protein is a metal-containing enzyme. Increasing the urea concentration to 4 M did not significantly change the enzyme activity, and even 5 M urea decreased the activity by only 34%. Among the reagents tested, DTT displayed the greatest inhibitory effect. Thus, in the presence of 0.01 M DTT the enzyme activity was only 54% of the maximum.

Similarly to other bilirubin oxidases, BOX is promising for medicine as an enzyme capable of oxidizing blood bilirubin, and its laccase activity markedly increases the range of its possible application. To confirm this, we used BOX for decolorization of the synthetic azo dye TB [30]. Trypan Blue (5 mg/liter) in aqueous solutions and buffer systems (pH 5.0) at 25°C was completely decolorizated within 3 h in the presence of BOX (20 mU/ml). Increasing the temperature to 30-40°C and/or addition of redox mediators, such as ABTS, increased tenfold the rate of TB decolorization [30].

DISCUSSION

The enzyme isolated from *P. ostreatus* mycelium was purified to homogeneity, and determination of its structural and kinetic parameters allowed us to characterize it as an intracellular fungal bilirubin oxidase (BOX; EC 1.3.3.5) with pronounced laccase features. The biosynthesis of BOX was induced during the initial period of the growth of the fungus, and the enzyme was produced during the fungus lifetime; therefore, the enzyme is supposed to be involved in biosynthesis of a necessary cellular component, e.g. pigments of the mycelium. This hypothesis was supported by a close association of the enzyme with the pigment, which could be separated only by affinity chromatography of the protein on ConA-Sepharose at very high concentration of ammonium sulfate in the buffer used for washing the sorbent. Intracellular laccases were shown to be involved in synthesis of melanin in the fungi *Lentinula edodes*, *Pycnoporus cinnabarinus*, and *Trametes pubescens* [31-33]. However, the ability of BOX to directly oxidize a wide variety of diphenol derivatives, organic heterocyclic compounds, dyes, and tetrapyrroles did not preclude the involvement of the isolated oxidase in detoxification of organic pollutants, and this is in agreement with some works [34, 35].

Although the biological role of the intracellular BOX is not yet clear, advantages of the enzyme use in practice are clear. The stability of BOX over a wide range of temperature and pH and easy conditions (25°C in water) of oxidizing many organic substances without production of toxic intermediates make the prepared enzyme promising for application in textile, cellulose-paper, food, and cosmetic industries, for detoxification and discoloration of waste water, degradation of xenobiotics and bioremediation, in elaboration of biosensors and cathodes of biofuel elements, etc. [3]. The ability of the enzyme to transform bilirubin suggests its application in medicine.

We are grateful to V. N. Gladyshev (Department of Biochemistry, University of Nebraska, Lincoln 68588-0664, USA) for his suggestion of performing ESI-MS-MS analysis and to N. M. Rovbel (Institute of Microbiology, National Academy of Sciences of Belarus) for presenting the white rot fungus *Pleurotus ostreatus* 43 ((Fr.) P. Kummer) BIM F-306D for inoculation.

This work was supported by the Ministry of Education of Belarus (project No. 2007-1944).

REFERENCES

1. Messerschmidt, A., and Huber, R. (1990) *Eur. J. Biochem.*, **187**, 341-352.
2. Enguita, F., Martins, J. L., Henriques, A. O., and Carrondo, M. A. (2003) *J. Biol. Chem.*, **278**, 19416-19425.
3. Morozova, O. V., Shumakovich, G. P., Gorbacheva, M. A., Shleev, S. V., and Yaropolov, A. I. (2007) *Biochemistry (Moscow)*, **72**, 1136-1150.
4. Baldrian, P. (2006) *FEMS Microbiol. Rev.*, **20**, 1-28.
5. Blaich, R., and Esser, K. (1975) *Arch. Microbiol.*, **103**, 271-277.

6. Hiromi, K., Yamaguchi, Y., Sugiura, Y., Iwamoto, H., and Hirose, J. (1992) *Biosci. Biotech. Biochem.*, **56**, 1349-1350.
7. Matsui, Y., Satoh, T., and Nakajima, K. (1984) *Jpn. Patent. Japan Kokai Tokkyo Koho*, 59–135886.
8. Uwajima, T., and Hamamatsu, M. (1984) *Jpn. Patent. Japan Kokai Tokkyo Koho*, 59–198971.
9. Xu, F., Shin, W., Brown, S. H., Wahleitner, J. A., Sundaram, U. M., and Solomon, E. I. (1996) *Biochim. Biophys. Acta*, **1292**, 303-311.
10. Tanaka, N., and Muran, S. (1983) *Agric. Biol. Chem.*, **47**, 1627-1628.
11. Coll, P. M., Fernandez-Abalos, J. M., Villanueva, J. R., Santamaria, R., and Perez, P. (1993) *Appl. Environ. Microbiol.*, **59**, 2607-2613.
12. Heinzkill, M., Bech, L., Halkier, T., Schneider, P., and Anke, T. (1998) *Appl. Environ. Microbiol.*, **64**, 1601-1606.
13. Masuda-Nishimura, I., Ichikawa, K., Hatamoto, O., Abe, K., and Koyama, Y. (1999) *J. Gen. Appl. Microbiol.*, **45**, 93-97.
14. Palmieri, G., Giardina, P., Bianco, C., Scaloni, A., Capassoi, A., and Sannia, G. (1997) *J. Biol. Chem.*, **272**, 31301-31307.
15. Bradford, M. M. (1976) *Anal. Biochem.*, **72**, 248-254.
16. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
17. Dawson, R., Elliott, D., Elliott, W., and Jones, K. (1991) *Data for Biochemical Research* [Russian translation], Mir, Moscow.
18. McIlvaine, T. C. (1921) *J. Biol. Chem.*, **49**, 183-186.
19. Murao, S., and Tanaka, N. (1981) *Agric. Biol. Chem.*, **45**, 2383-2384.
20. Garcia, T. A., Santiago, M. F., and Ulhoa, C. J. (2007) *Appl. Microbiol. Biotechnol.*, **75**, 311-318.
21. Pozdnyakova, N. N., Turkovskaya, O. V., Yudina, E. N., and Rodakevich-Novak, Ya. (2006) *Prikl. Biokhim. Mikrobiol.*, **42**, 63-69.
22. Mansur, M., Arias, M., Copa-Patino, J., Flardh, M., and Gonzalez, A. (2003) *Mycologia*, **95**, 1013-1020.
23. Tanaka, H., and Murao, S. (1982) *Agric. Biol. Chem.*, **46**, 2499-2503.
24. Galhaup, C., Goller, S., Peterbauer, C., Strauss, J., and Haltrich, D. (2002) *Microbiology*, **148**, 2159-2169.
25. Kanunfre, C. C., and Zancan, G. T. (1998) *FEMS Microbiol. Lett.*, **161**, 151-156.
26. Rogalski, J., Dawidowicz, A. L., and Leonowicz, A. (1990) *Acta Biotechnol.*, **10**, 261-269.
27. Shleev, S. V., Morozova, O., Nikitina, O., Gorshina, E. S., Rusinova, T., Serezhenkov, V. A., Burbaev, D. S., Gazaryan, I. G., and Yaropolov, A. I. (2004) *Biochimie*, **86**, 693-703.
28. Vares, T., and Hatakka, A. (1997) *Can. J. Bot.*, **75**, 61-71.
29. Sakasegawa, S., Ishikawa, H., Imamura, S., Sakuraba, H., Goda, S., and Ohshima, T. (2006) *Appl. Microbiol. Biotechnol.*, **72**, 972-975.
30. Pakhadnia, Yu. G., and Lapko, A. G. (2008) *Vestsi NAN Belarusi*, *Ser. Biol. Navuk*, **5** (Suppl.), 211-215.
31. Nagai, M., Kawata, M., Watanade, H., Ogawa, M., Saito, K., Takesawa, T., Kanda, K., and Sato, T. (2003) *Microbiology*, **149**, 2455-2462.
32. Eggert, C., Temp, U., Dean, J. F. D., and Eriksson, K.-E. L. (1995) *FEBS Lett.*, **376**, 202-206.
33. Galhaup, C., and Haltrich, D. (2001) *Appl. Microbiol. Biotechnol.*, **56**, 225-232.
34. Kakaoka, K., Tanaka, K., Sakai, Y., and Sakurai, T. (2005) *Protein Exp. Purif.*, **44**, 77-83.
35. Zhang, X., Liu, Y., Yan, K., and Wu, H. (2007) *J. Biosci. Bioeng.*, **104**, 104-110.