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Extracellular Oxidases of the Lignin-Degrading Fungus *Panus tigrinus*

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Abstract—Two extracellular oxidases (laccases) were isolated from the extracellular fluid of the fungus *Panus (Lentinus) tigrinus* cultivated in low-nitrogen medium supplemented with birch sawdust. The enzymes were purified by successive chromatography on columns with TEAE-cellulose and DEAE-Toyopearl 650M. Both oxidases catalyze oxidation of pyrocatechol and ABTS. Moreover, oxidase 1 also catalyzes oxidation of guaiacol, *o*-phenylenediamine, and syringaldazine. The enzymes have identical pH (7.0) and temperature (60-65°C) optimums. Absorption spectra of the oxidases differ from the spectra of typical "blue" laccases and are similar to the spectrum of yellow oxidase.

Key words: lignin, biodegradation, oxidase, laccase, white rot fungi, *Panus (Lentinus) tigrinus*

Lignin is a complicated three-dimensional nonstereoregular polymer widely distributed in nature. Lignin is degraded by Basidiomycetes (called white rot fungi) with involvement of a complex of extracellular oxidizing enzymes, mainly peroxidases and oxidases [1, 2]. The ratio and role of individual enzymes varied in different fungi, but all known lignin-degrading fungi secrete laccase (a copper-containing oxidase).

Laccase is interesting because of its involvement in destruction of lignin and the possibility of its application in industry [3, 4]. Modification of wood wastes with the white rot fungus *Panus tigrinus* makes possible production of pressed wood materials [5, 6]. This modification is caused by the high lignin-degrading activity of the fungus. Laccase and Mn peroxidase seem to play a crucial role in biodegradation of wood lignin [7]. A secretory plant-type peroxidase was isolated from a submerged culture of the fungus and characterized [7]. The properties of the laccase were not investigated. But it has been suggested [8, 9] that *P. tigrinus* secretes some extracellular oxidases with unusual features. The purpose of the present work was to isolate the extracellular laccase of *P. tigrinus* and study features of this enzyme.

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MATERIALS AND METHODS

Pyrocatechol, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt (ABTS), guaiacol, syringaldazine, *o*-phenylenediamine, and Remazol Brilliant Blue R (RBBR) were from ICN (USA), bovine serum albumin was from BDH Biochemicals (England), TEAE-cellulose was from Reanal (Hungary), DEAE-Toyopearl 650M was from Toyo Soda (Japan), and Coomassie Brilliant Blue was from Loba Feinchemie (Czechia). Other reagents were of domestic production.

Cultivation and determination of the enzymatic activity. The fungus *Panus (Lentinus) tigrinus*, strain BKM F-3616 D [10] was cultivated at 26°C in 500-ml Erlenmeyer conical flasks containing 100 ml of nutrient medium supplemented with birch sawdust for 6-8 days on circular shakers (235 rpm) as described in [7, 11]. The culture fluid was centrifuged at 4000*g* for 10 min. The supernatant was used for determination of the laccase activity by oxidation of pyrocatechol at 410 nm (ε = 0.740 $mM^{-1} \cdot cm^{-1}$) [12]. The initial rate of the reaction was measured with an SF-46 spectrophotometer (LOMO, Russia). The enzyme amount catalyzing oxidation of 1 μmol substrate per min under optimal conditions was taken as the activity unit. The protein concentration

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was determined spectrophotometrically by the Bradford method with BSA as the standard [13].

Purification of the enzyme. The enzyme was purified using our modification of the conventional method [14]. The culture fluid of the fungus (0.5 liter) was taken at the maximum laccase activity on the 8th day, filtered, and precipitated with stirring in the presence of 90% ammonium sulfate. The precipitate from centrifugation at 2500*g* for 30 min was dissolved in distilled water. After dialysis for 24 h against 5 mM potassium phosphate buffer (pH 6.0), the preparation was concentrated to the volume of 50-60 ml by maintaining in dextran at 4°C. The concentrated preparation was applied onto a column with TEAE-cellulose and eluted with 1 M NaCl. Fractions with the laccase activity were combined and subjected to chromatography on a column with DEAE-Toyopearl 650M (2 × 20 cm) equilibrated with 5 mM potassium phosphate buffer (pH 7.2). The protein was eluted with a linear gradient (from 5 to 200 mM) of potassium phosphate buffer (2 × 250 ml, pH 7.2). The elution rate was 3 ml/min.

Characteristics of the enzymes. The pH optimum (at 30°C) and temperature optimum of the combined fractions were determined after each stage of chromatography by oxidation of pyrocatechol in 5 mM potassium phosphate buffer. The substrate specificity of the enzyme was determined using ABTS [15], guaiacol, *o*-phenylenediamine [16], RBBR, pyrocatechol [12], and syringaldazine [17]. The reaction mixture contained 0.1 mM substrate, 0.5 ml of the enzyme solution, and 2.4 ml of the appropriate buffer. Isoelectric points of the enzymes were determined by the pH value corresponding to the greatest clarity of the enzyme solution. The absorption spectra of the laccases were recorded in 5 mM potassium phosphate buffer (pH 6.0) with a Specord M-40 spectrophotometer (Germany).

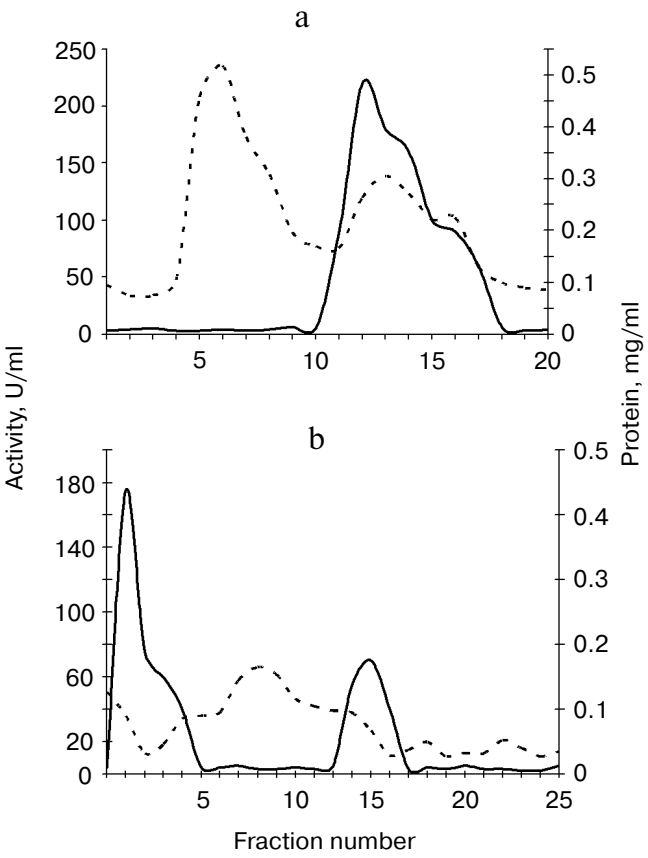

Fig. 1. Chromatography of laccase from *P. tigrinus* on TEAE-cellulose (a) and DEAE-Toyopearl 650M (b). Solid and dashed lines designate activity and protein, respectively.

RESULTS AND DISCUSSION

Results of purification of the peroxidases are presented in Table 1. The yield of oxidase (by oxidation of pyrocatechol) after purification on TEAE-cellulose was 47% of the baseline activity. This was associated with a more than 16-fold increase in the specific activity. Results of chromatography of the proteins on TEAE-cellulose are shown in Fig. 1a. Laccase was eluted mainly in fractions 10-17.

Rechromatography of active fractions of laccase on DEAE-Toyopearl resulted in two distinctly separated peaks of the activity by pyrocatechol (Fig. 1b), which we

Table 1. Purification of oxidases from *P. tigrinus*

Stage of purification	Volume, ml	Protein, mg/ml	Specific activity, U/mg	Yield, %
Culture fluid	500	0.15	139	100
Chromatography on TEAE-cellulose	21	0.10	2320	47
Chromatography on DEAE-Toyopearl 650M				
Oxidase 1	12	0.08	2190	21
Oxidase 2	9	0.07	750	5

denote as oxidase 1 and oxidase 2. The yield was 21 and 5%, respectively. The specific activity of oxidase 1 was slightly lower than after the elution from TEAE-cellulose but 15-fold higher than in the culture fluid.

After chromatography on TEAE-cellulose and DEAE-Toyopearl, the active fractions were combined and used for investigating the enzyme features. The combined active fractions of the enzyme from TEAE-cellulose (Fig. 2) and oxidases 1 and 2 displayed the maximum laccase activity with pyrocatechol as a substrate at 30°C at the pH optimum of 7.0 (Fig. 2a). The temperature optimum for laccase was 65°C (Fig. 2b). Values of p*I* for oxidases 1 and 2 were 4.19 and 3.95, respectively.

The substrate specificities of the isolated oxidases were studied, and oxidase 1 oxidized pyrocatechol, guaiacol, *o*-phenylenediamine, ABTS, and syringaldazine, and the activity was maximal with pyrocatechol as a substrate. Oxidase 2 oxidized only ABTS and pyrocatechol (Table 2).

The absorption spectra of the isolated oxidases after chromatography on TEAE-cellulose (Fig. 3a) have maximums at 280 and 330-340 nm. Copper ions in proteins are subdivided into three types on the basis of their spectral features. Copper ions of the type I absorb at 600-610 nm and are responsible for an intense blue color of the proteins which contain them. Spectral characteristics of Cu^{2+} of type II are similar to low-molecular-weight copper complexes which are of a light blue color due to (*d*–*d*)-transitions in the region of 660-800 nm of the absorption spectrum of Cu^{2+} [18]. As differentiated from Cu^{2+} of types I and II, copper ions of type III have some specific characteristics caused by features of their ligands. These ions exist as pairs with mutually compensated spins that prevents their contribution to the EPR spectrum, but they have a specific absorption band with maximum at 330-340 nm [19, 20]. Copper ions of types I and II are absent in the isolated laccases, but they contains two copper ions of type III as a binary complex [21, 22].

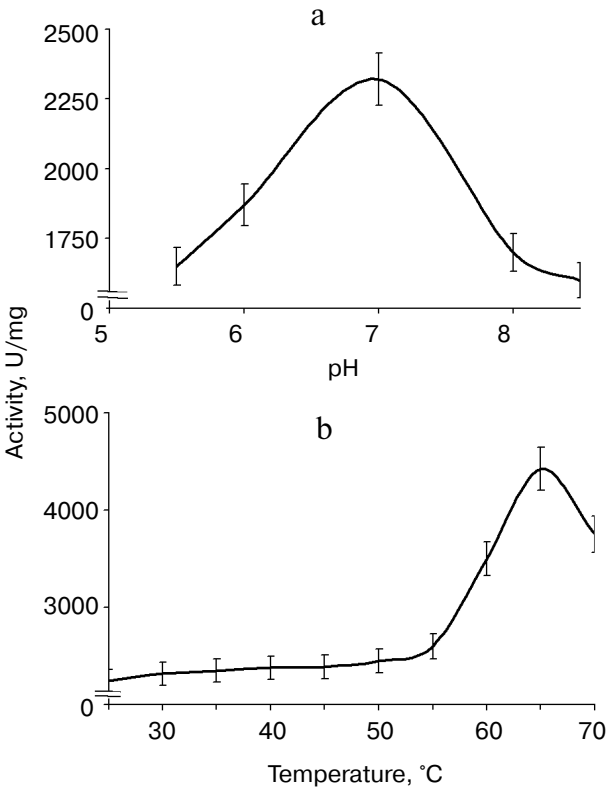

Fig. 2. Effects of pH (a) and temperature (b) on activity of laccase from *P. tigrinus* with pyrocatechol as a substrate.

The spectrum obtained by us was compared with the absorption spectra of yellow oxidase from the fungus

Table 2. Activities of oxidases from *P. tigrinus* after purification on TEAE-cellulose and DEAE-Toyopearl 650M determined with varied substrates

Substrate	Wavelength at determination, nm	Molar absorption coefficient of the product, $mM^{-1}\cdot cm^{-1}$	Buffer	Activity, % of the activity with ABTS	
				oxidase 1	oxidase 2
ABTS	436	29.8	Sodium acetate, pH 5.0	100*	100*
Guaiacol	470	5.57	Potassium phosphate, pH 7.0, 0.05% $CaCl_2$	700	0
o-Phenylenediamine	445	11.1	Sodium acetate, pH 5.0	300	0
Syringaldazine	525	65.0	Sodium acetate, pH 5.5	100	0
Pyrocatechol	410	0.74	Potassium phosphate, pH 7.0	3770	2690
RBBR	595	6.90	Sodium acetate, pH 4.5	0	0

* The specific activities of oxidases 1 and 2 with ABTS as a substrate were 58 and 28 U/mg protein, respectively.

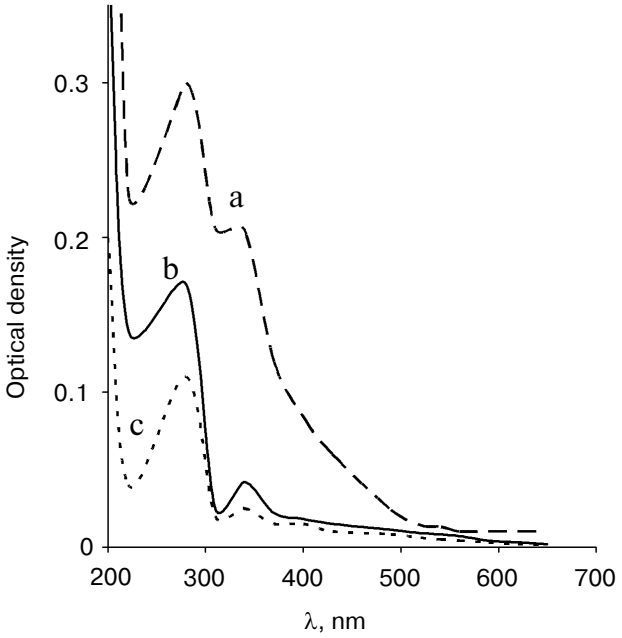

Fig. 3. Absorption spectra of laccases from *P. tigrinus* isolated by chromatography on TEAE-cellulose (a) and of oxidases 1 (b) and 2 (c) in 5 mM potassium phosphate buffer (pH 6.0).

Panus tigrinus 8/18 and of laccase from *Coriolus versicolor* BKM F-116 [8, 9, 23], and this revealed the absence of the specific absorption maximum at 610 nm in the spectra of both our preparation and yellow oxidase. This maximum is specific for blue laccases and suggests the presence of a "blue" copper metal center of type I. Thus, the laccase isolated by us should be a yellow oxidase. According to the above-mentioned works, a contact of the reduced enzyme with molecular oxygen can restore the initial appearance of the absorption spectrum with the maximum at 610 nm.

It seems that reactive products of lignin degradation can reduce Cu^{2+} in laccase. Primary products of lignin degradation caused by fungi are phenols, quinones, and aromatic radicals, such as phenoxy radicals and cation radicals. In this case, the purified enzyme should not have the absorption spectrum with the maximum at 610 nm (the "blue" atom of the type I copper center is reduced). Although this hypothesis by Leontievskii and Pozdnyakova casts some doubt [24], nevertheless, it explains not only unusual features of oxidase from *P. tigrinus* but also the existence of other atypical "not blue" laccases, e.g., yellow-brown laccase from *Agaricus bisporus* [25] also isolated from a solid-phase culture, white laccase from *Pleurotis ostreatus* [26], or the laccase isolated by us from the fungus *Panus tigrinus* BKM F-3616 D.

The absorption spectra of oxidases 1 and 2 (Figs. 3b and 3c, respectively) were similar in the range of 200-700 nm and presented usual protein spectra with the absorption maximum at 280 nm. There were no maximums characteristic for typical blue oxidases (610 nm), flavin oxidases (360 and 460 nm), or heme-containing peroxidases (390-420 nm).

Our findings suggest that oxidase 2 is an isoform of oxidase 1, which seems to be a yellow laccase. Oxidase 1 catalyzes oxidation of syringaldazine and pyrocatechol, which are used for testing the laccase character of oxidases [8, 12].

Thus, we have isolated from the fungus *P. tigrinus* two atypical oxidases different in substrate specificity but possessing identical values of pH and temperature optimums and absorption spectra in the range of 200-700 nm not characteristic for blue laccases.

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