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# Oxidase of the white rot fungus *Panus tigrinus* 8/18

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**Abstract**

Extracellular oxidase of the white rot fungus *Panus tigrinus* (earlier reported as laccase) contains copper but has no absorption spectrum typical of 'blue' oxidases. Thioglycolate and sodium azide inhibit the activity of this enzyme at concentrations 2.5–3 orders lower than those needed for fungal laccases. The oxidase of *P. tigrinus* oxidizes syringaldazine, coniferyl alcohol, ABTS, syringic acid, diaminobenzidine, guaiacol, catechol and vanillylacetone with different efficiencies. Oxygen consumption and no hydrogen peroxide formation were detected during substrate oxidation by *P. tigrinus* oxidase. It is proposed that *P. tigrinus* oxidase is a new ligninolytic enzyme.

*Key words:* Oxidase; Copper enzyme; White rot fungus; Lignin biodegradation

## 1. Introduction

White rot fungi degrade lignin with the participation of extracellular enzymes, commonly including lignin peroxidase, Mn-dependent peroxidase and laccase [1].

The ligninolytic activity of laccase, a 'blue' copper oxidase, is not considered to be high: being a phenol oxidase, it is unable to oxidize non-phenolic substructures predominant in lignin molecules [2].

The lignin-degrading white rot fungus *Panus tigrinus* produces a complex of extracellular ligninolytic enzymes, which contains an oxidase, presumed to be of the laccase type [3,4]. The general properties of the *P. tigrinus* oxidase, determined by us earlier [3], resemble those of known fungal laccases. However, the enzyme's ability to split the $C_\alpha$–$C_\beta$ bond in a non-phenolic $\beta$-1 type of lignin model compound [4,5] raised the question of its nature.

## 2. Materials and methods

### 2.1. Enzyme production and purification

The white rot fungus *P. tigrinus* 8/18 isolated from rotten wood in Dushanbe, Tadzhikistan, was used. The fungus was grown under conditions of solid-phase fermentation of wheat straw [3]. Oxidase purification and testing of its homogeneity were as described earlier [3]. Oxidase activity was assayed by syringaldazine oxidation [6]. Protein concentration was measured by staining with Coomassie brilliant blue G-250 [7]. The purified enzyme was concentrated by ultrafiltration on a PM-10 membrane (Amicon).

### 2.2. Spectral properties

The oxidase absorption spectrum was recorded using a Shimadzu UV-160 spectrophotometer within a range of 200–800 nm in 20 mM Na-acetate buffer (pH 5.0); the protein concentration was 0.45 mg/ml.

The content of copper, iron and zinc in the enzyme preparation was determined by a Perkin-Elmer 5100/Zeeman atomic absorption spectrometer.

### 2.3. Enzymatic reactions

Oxidase substrate specificity was determined spectrophotometrically (Shimadzu UV-160) as the rate of substrate consumption or product formation in 20 mM Na-acetate buffer at pH 5.0, 25°C (pH 4.5, at vanillylacetone oxidation). As substrates, we used *o*-, *m*- and *p*-cresols (Serva); catechol (Sigma) [8]; veratryl alcohol (Sigma) [9]; syringaldazine (Serva) [6]; diaminobenzidine, syringic acid (Sigma); guaiacol, sinapic acid (Reakhim, Russian Federation); coniferyl alcohol (Serva); vanillylacetone [10]; 2,2-azinobis-3-ethylbenzthiazolinsulfonic acid (Fluka) [11]; anisyl alcohol, veratryl acid (Reakhim), homoveratryl acid (Serva) [12]; Phenol red (Serva) [13]. Enzyme activity with phenol and 4-chlorophenol (Serva) was assayed by the change in the absorption spectrum of the reaction mixture. Vanillylacetone was synthesized according to Huynch et al. [14].

Enzyme activity was expressed in conventional units equal to absorption change at a corresponding wavelength per 1 min for 1 mg of oxidase enzyme preparation.

Effect of inhibitors was determined after 15 min preincubation of the reaction mixture without substrate at 25°C.

### 2.4. Oxygen consumption

Oxygen consumption during oxidation of coniferyl alcohol, syringaldazine and ABTS by oxidase was estimated using an oxygen electrode and LP-7 polarograph (Czechoslovakia) at room temperature. To detect hydrogen peroxide, 200 U of catalase (Sigma) were added when the oxygen consumption was over; 0.6 mg of horseradish peroxidase (Serva) was supplemented to the reaction mixture containing 20 mM Na-acetate buffer, the substrate and oxidase to detect $H_2O_2$ spectrophotometrically: in the case of $H_2O_2$ formation, the rate of substrate oxidation was expected to increase due to simultaneous peroxidase action.

## 3. Results and discussion

The AAS showed that *P. tigrinus* oxidase contains copper and does not contain zinc or iron. There is no absorption spectrum typical of 'blue' copper proteins, including laccases (absorption bands near 300 and 600 nm) [15]. The absence of an absorption spectrum typical of 'blue' oxidases indicates that copper atoms of the *P. tigrinus* oxidase may have different surroundings compared to that in fungal laccases.

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*Abbreviations:* ABTS, 2,2′-azinobis-(3-ethylbenzothazoline-6-sulfonic acid); EDTA, ethylenediaminetetraacetic acid; AAS, atomic absorption spectroscopy.


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Table 1
Effect of inhibitors on ABTS oxidation by *P. tigrinus* oxidase

| Inhibitor | Concentration (mM) | Oxidase activity (%) |
|---|---|---|
| Control | – | 100.0 |
| Sodium azide | 0.0002 | 78.1 |
| | 0.0008 | 52.4 |
| | 0.002 | 25.1 |
| | 0.006 | 0 |
| EDTA | 0.1 | 95.5 |
| | 1.0 | 58.6 |
| | 2.0 | 43.6 |
| | 4.0 | 43.3 |
| | 5.0 | 42.6 |
| Thioglycolate | 0.0002 | 85.2 |
| | 0.001 | 59.5 |
| | 0.002 | 34.2 |
| | 0.004 | 0 |
| Hydroxylamine | 0.8 | 100.0 |
| | 1.0 | 94.0 |
| | 5.0 | 74.4 |
| β-Mercaptoethanol | 0.002 | 92.6 |
| | 0.008 | 80.7 |
| | 0.03 | 50.0 |
| | 0.05 | 26.8 |
| | 0.06 | 0 |
| Carbon monoxide | blow out: 3 min | 62.0 |
| | blow out: 6 min | 24.5 |
| | blow out: 9 min | 0 |

Nonetheless, these atoms participate in the catalytic process since thioglycolate, a specific copper chelator, and EDTA, a non-specific chelator of two-valent metals, inhibit the enzyme (Table 1). It is noteworthy that sodium azide and thioglycolate are efficient at concentrations 2.5–3 orders lower than in the case of fungal laccases [16]. EDTA concentrations higher than 2 mM cause no further decrease in the oxidase activity: possibly, the enzyme has catalytically significant atoms of metals, for instance, copper atoms inaccessible to the action of this inhibitor.

Table 2
Compounds tested as substrates of *P. tigrinus* oxidase

| Substrate | $V_m$ | $K_m$ ($\times 10^{-6}$ M) | $V_m/K_m$ |
|---|---|---|---|
| Syringaldazine | 588.24 | 7.69 | 76.49 |
| Coniferyl alcohol | 714.29 | 15.39 | 46.41 |
| ABTS | 28.72 | 33.40 | 8.58 |
| Syringic acid | 133.00 | 20.00 | 6.65 |
| Diaminobenzidine | 250.00 | 500.00 | 0.50 |
| Guaiacol | 45.50 | 746.23 | 0.06 |
| Catechol | 57.14 | 4760.00 | 0.01 |
| Vanillylacetone | 714.28 | 15.38 | 46.44 |
| Phenol | | no oxidation | |
| 4-Chlorophenol | | – | |
| Cresol (*o*-, *m*-, *p*-) | | – | |
| Phenol red | | – | |
| Anisyl alcohol | | – | |
| Veratryl alcohol | | – | |
| Veratric acid | | – | |
| Homoveratric acid | | – | |

Table 3
Coupled action of the *P. tigrinus* oxidase and horseradish peroxidase

| Reaction mixture | Activity (conventional units) |
|---|---|
| 1. Horseradish peroxidase + syringaldazine | 0 |
| 2. Horseradish peroxidase + syringaldazine + hydrogen peroxide | 0.3 |
| 3. *P. tigrinus* oxidase + syringaldazine | 1.7 |
| 4. *P. tigrinus* oxidase + syringaldazine + horseradish peroxidase | 1.6 |
| 5. *P. tigrinus* oxidase + syringaldazine + horseradish peroxidase + hydrogen peroxide | 2.0 |

The results presented in Table 2 indicate that the range of substrate specificity of *P. tigrinus* oxidase in general corresponds to that for common fungal laccases. The most remarkable exception is the reaction with vanillylacetone. To date, this substrate has been known as a specific reagent for Mn-peroxidase; other ligninolytic enzymes (lignin peroxidase and laccase) are not capable of oxidizing this compound [17].

The substrate oxidation by *P. tigrinus* oxidase was accompanied by consumption of oxygen in the reaction mixture. Addition of catalase to the reaction mixture on completion of the reaction failed to induce evolution of oxygen. Introduction of horseradish peroxidase did not increase the substrate oxidation rate (Table 3). These results show that oxygen is not reduced to $H_2O_2$ during the oxidation of substrate by *P. tigrinus* oxidase.

The substrate specificity and the results of the inhibitor analysis of *P. tigrinus* oxidase in some respects resemble the properties of fungal laccases. However, the spectral analysis data, oxidation of the dimeric non-phenolic model compounds of lignin [4,5] and vanillylacetone do not allow one to attribute it to laccase or 'blue' oxidases. It could be neither tyrosinase (no cresolase activity, the activity is inhibited by CO) nor flavin-containing oxidase (no reduction of oxygen to hydrogen peroxide; unlikely, in the case of arylalcoholoxidase, there is no oxidation of aromatic alcohols). Research into elucidating the nature of this enzyme is still in progress.

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