FEBS Letters 413 (1997) 446–448 FEBS 19101

'Yellow' laccase of *Panus tigrinus* oxidizes non-phenolic substrates without electron-transfer mediators

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Received 10 July 1997

Abstract Yellow and blue forms of laccase from solid-state and submerged cultures of *Panus tigrinus* were isolated. Both laccases had similar molecular masses and specific activity, but yellow laccase had no 'blue' maximum in the absorption spectrum. Blue laccase oxidized veratryl alcohol and a non-phenolic dimeric lignin model compound only in the presence of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) as electron-transfer mediator. Yellow laccase catalyzed these reactions without any additional compounds. It is supposed that yellow laccase is formed as a result of blue laccase modification by products of lignin degradation. These compounds might play a role of natural electron-transfer mediators for the oxidation of non-phenolic substances, catalyzed by yellow laccase.

Key words: White rot fungus; Laccase; Redox-mediator; Absorption spectra; Lignin

1. Introduction

Laccase (EC 1.10.3.2; benzenediol:oxygen oxidoreductase) is an extracellular blue oxidase distributed in plants and fungi. It contains four copper atoms, which are organized into three types of copper sites and determine characteristic spectral properties of the enzyme. All copper atoms take part in the catalytic process of oxygen reduction to water [1–3]. Laccase production is a typical property of ligninolytic wood rot fungi. The ligninolytic potential of fungal laccases was evaluated rarely because laccase does not oxidize non-phenolic structures which are predominant in lignin. However, it was found that the *Coriolus versicolor* laccase oxidized non-phenolic compounds in the presence of aromatic electron-transfer mediators [4].

In our laboratory, H_2O_2- and Mn^{2+}-independent activity capable of oxidizing a non-phenolic dimeric lignin model compound was found in the extracellular enzyme preparation isolated from a solid-state culture of the ligninolytic fungus *Panus tigrinus* [5]. Then, the corresponding oxidase was purified from this preparation. Unlike typical blue laccase, this enzyme had yellow-brown color, had no 'blue' maximum in the optical absorption spectrum, had no typical EPR spectrum, was inhibited by CO and oxidized a non-phenolic dimeric lignin model compound [6–9]. This permits us to suggest that the enzyme from *P. tigrinus* is a new type of fungal oxidases [8]. However, it was found later that yellow enzyme from *P. tigrinus* contains four copper atoms per mole of protein and catalyzes the four-electron reduction of oxygen to water, as usual laccase. Study of the substrate specificity and the effect of inhibitors also confirmed the laccase nature of this enzyme. Taking into account that the yellow oxidase from *P. tigrinus* was isolated from a solid-state culture on wheat straw containing lignin we suggested that the unusual properties of this enzyme are the result of laccase modification by reactive products of lignin degradation [9].

This work was designed to compare the oxidation of non-phenolic compounds by yellow laccase from a lignin-containing solid-state culture of *P. tigrinus* and blue laccase from a submerged culture of *P. tigrinus* without lignin.

2. Materials and methods

2.1. Enzyme production and purification

The white rot fungus *P. tigrinus* 8/18 was isolated from rotten wood in Dushanbe (Tadjikistan). The fungus was grown under conditions of solid-state fermentation of wheat straw [10] and in submerged culture on N-limited liquid mineral medium with 1% glucose as carbon source and 2 mM *m*-methylbenzylic alcohol as laccase inducer [11]. Laccase from both types of cultures was purified according to method described earlier [10]. Laccase activity was assayed by the rate of ABTS oxidation in 20 mM sodium acetate, pH 5.0 (Buffer A) [8], specific activity expressed in arbitrary units as $\Delta A_{436} \cdot min^{-1} \cdot mg^{-1}$. Protein concentration was determined with Coomassie blue.

2.2. Electrophoresis

SDS-PAGE [12] was performed in 12% gel. Protein MM standards (Pharmacia) were (kDa): phosphorylase *b*, 94; bovine serum albumin, 67; ovalbumin, 43; carbonic anhydrase, 30; soybean trypsin inhibitor, 20.1; α-lactalbumin, 14.4.

2.3. Absorption spectra

UV/visible absorption spectra of laccases (1 mg/ml) were recorded on a Shimadzu UV-160 spectrophotometer in Buffer A.

2.4. Oxidation of non-phenolic compounds

Reaction mixtures (3 ml) contained Buffer A, 3 mM veratryl alcohol or 2 mM β-1 type model compound of lignin, 7 μg/ml laccase and, if necessary, 0.8 mM ABTS. Reaction mixtures were filtered through a 0.2 μm pore-size filter with low protein-binding capacity into sterile 25-ml flasks, flushed by sterile oxygen for 10 min, plugged and incubated at 37°C for 24 or 48 h.

Reaction products were extracted with ethylacetate and developed by TLC [6]. Formation of veratraldehyde was confirmed by TLC, UV/visible absorption spectrum (Shimadzu UV-160, ethanol solution) and MS (8430 Finnigan Mat, MS *m/z*: 166 (M^+, 100%), 165 (69), 151 (17), 137 (10), 95 (32.6), 79 (16.3), 77 (26.6)).

2.5. Chemicals

ABTS, veratryl alcohol and veratraldehyde were obtained from Aldrich Chemical Co. 1,2-bis(3,4-dimethoxy phenyl)-1,3-propanediol (lignin dimeric model compound of β-1 type) was synthesized by Dr. V.P. Shevchenko (IBPM RAS).

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Abbreviations: ABTS, 2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); AAS, atomic absorption spectroscopy; EPR, electron paramagnetic resonance; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; TLC, thin layer chromatography

PII S0014-5793(97)00953-8

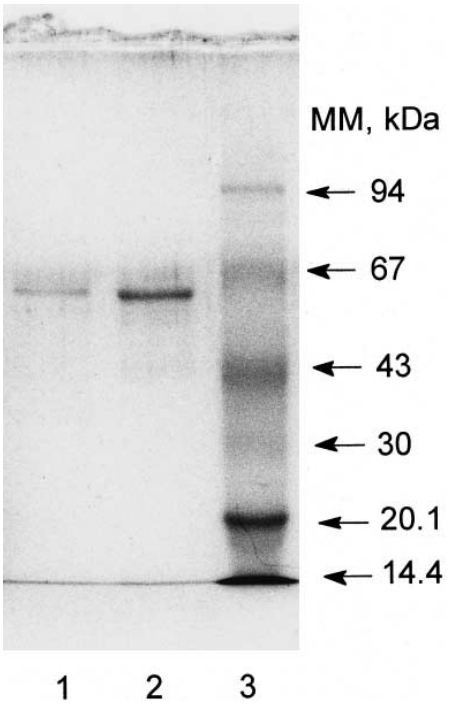

Fig. 1. SDS-PAGE of blue and yellow laccases of *P. tigrinus*. Lane 1: yellow laccase; lane 2: blue laccase; lane 3: protein MM markers.

3. Results

3.1. Enzyme purification

P. tigrinus laccases were isolated from the liquid medium of submerged cultures, grown for 9 days and from extracts of wheat straw fermented during 5 days. Enzymes purified from both kinds of cultures had equal MM (63 kDa, Fig. 1) and similar values of specific activity (Table 1). Laccase from submerged culture was blue (1 mg/ml) and had a typical absorption spectrum with 610 nm 'blue' maximum, A_{280}/A_{610}=30. Laccase from solid-state culture had yellow-brown color (1 mg/ml) and no 'blue' maximum in the absorption spectrum (Fig. 2 and Table 1).

3.2. Oxidation of non-phenolic substrates catalyzed by blue and yellow laccases of P. tigrinus

Veratryl alcohol and a β-1 type dimeric model compound of lignin were used as the substrates for yellow and blue laccases.

Blue laccase did not oxidize these compounds in reaction mixture containing buffer, substrate and enzyme. Oxidation of both substrates was observed when the reaction mixture was supplemented with 0.8 mM ABTS as an electron-transfer mediator. After 24 h of incubation, veratraldehyde was found as a product of oxidation of these compounds (Fig. 3). Yellow laccase oxidized both veratryl alcohol and β-1 type dimeric model compound of lignin with formation of veratryl aldehyde in the absence of ABTS in the reaction mixture. No products of the reaction were found in reaction mixtures without enzymes or when laccases were boiled for 20 min prior addition to reaction mixture.

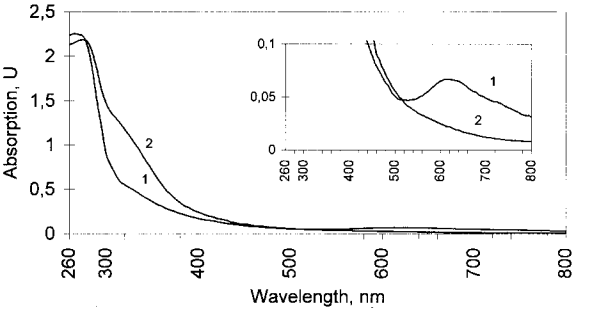

Fig. 2. Absorption spectra of blue and yellow laccases of *P. tigrinus*. Lane 1: blue laccase; lane 2: yellow laccase. 20 mM sodium acetate buffer, pH 5.0, 25°C; protein concentration 1 mg/ml.

4. Discussion

The primary products of the enzymatic oxidation of lignin by fungi are reactive compounds of radical nature, phenols, and quinones [13,14]. The probability of binding of such compounds to amino acid residues of the polypeptide chain of ligninolytic enzymes has been already discussed [15]. The unusual properties of yellow laccase from *P. tigrinus* purified from a solid-state culture suggest its probable modification by the products of lignin degradation. To check this suggestion, we purified the *P. tigrinus* laccase from a submerged lignin-free culture. In contrast to the yellow laccase isolated from a lignin-containing solid-state culture, the enzyme isolated from the submerged culture proved to be a typical blue laccase with the absorption maximum at 610 nm (Fig. 2). The molecular masses of blue and yellow laccases of *P. tigrinus* were found to be identical, whereas their specific activities were of the same order of magnitude. In the yellow laccase, the absence of a 'blue' maximum in its absorption spectrum (Fig. 2) and no EPR spectrum characteristic of blue laccases [9] resemble the properties of artificially reduced blue laccases [16,17]. We presume that the yellow laccase forms as a result of binding of aromatic products of lignin degradation with the blue laccase. A consequence of this modification may be the reduction of copper atoms of types 1 and 2 in the active center of the enzyme. The copper atoms may be reduced directly by the aromatic product of lignin degradation or their reduction may be mediated by an appropriate amino acid residue of the enzyme polypeptide chain owing to conformational changes resulting from the enzyme binding with molecules of a modifier which is a product of lignin degradation. The modifying agent, which is bound to the enzyme and reduces copper in its active center, may be reversibly substituted upon the enzyme saturation with substrate. Changes in the protein conforma-

Table 1
Properties of blue and yellow laccases of *P. tigrinus*

P. tigrinus laccase	Type of culture	MM (kDa)	Specific activity (arb. U)	Absorption maximum at 610 nm
Blue	Submerged	63	160	+
Yellow	Solid-state	63	199	-

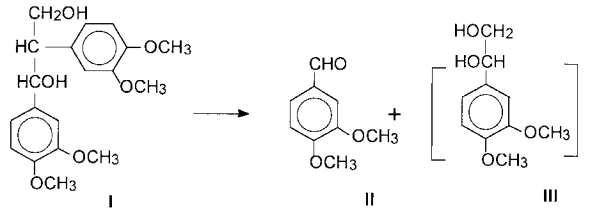

Fig. 3. Reaction of *P. tigrinus* blue laccase/ABTS couple and *P. tigrinus* yellow laccase with dimeric model compound of lignin of β-1 type. I: 1,2-bis(3,4-dimethoxy phenyl)-1,3-propanediol; II: veratraldehyde; III: veratryl glycol.

tion may explain the sensitivity of the yellow laccase from *P. tigrinus* to CO and its increased sensitivity to other inhibitors [8,9].

In the presence of aromatic electron-transfer mediators (e.g. ABTS), the blue laccase from *C. versicolor* becomes capable of oxidizing non-phenolic substrates [4]. The same effect was exerted by the laccase from *Picnoporus cinnabarinus* in the presence of the fungal metabolite 3-hydroxyanthranilate [18]. We reported earlier the first evidence of the capability of yellow laccase from *P. tigrinus* to oxidize non-phenolic substrates without exogenous addition of mediators [5–7]. The results presented in this paper support the idea of the presence of a lignin-generated aromatic modifier in the structure of yellow laccase [9]. Apparently, the modifier molecule bound to the apoenzyme of yellow laccase performs the function of electron-transfer mediator analogous to the role of ABTS or other compounds in the reaction of the blue laccase/mediator pair with non-phenolic substrates. The ability of yellow laccase to react with non-phenolic substrates explains the existence of fungal ligninolytic systems without lignin peroxidase.

Acknowledgements: The work was supported by Russian Ministry of Science and Technology Program 'Novel Methods in Bioengineering (Environmental Biotechnology)' and INTAS Grant No. 94-1195.

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