Biotechnology Letters (2006) 28: 633–636
DOI 10.1007/s10529-006-0026-3

Properties of laccases produced by *Pycnoporus sanguineus* induced by 2,5-xylidine

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Received 4 November 2005; Revisions requested 30 November 2005; Revisions received 26 January 2006; Accepted 30 January 2006

Key words: isoforms characterization, laccase, *Pycnoporus sanguineus*

Abstract

Two isoforms of laccase produced from the culture supernatant of *Pycnoporus sanguineus* were partially purified by phenyl-Sepharose chromatography. Molecular masses of the enzymes were 80 kDa (Lac I) and 68 kDa (Lac II). Optimum activity of Lac I was at pH 4.8 and 30 °C, and Lac II was at pH 4.2 and 50 °C over 5 min reaction. The K_m values of enzymes toward syringaldazine were 10 μM (Lac I) and 8 μM (Lac II). Sodium azide inhibited Lac I (85%) and Lac II (75%) activities.

Introduction

Laccase (benzenediol:oxygen oxidoreductase (EC 1.10.3.2) is a copper-containing enzyme that catalyzes the oxidation of a phenolic substrate by coupling it to the reduction of O_2 to water. Their function in fungi is varied and includes regulation of morphology, control of virulence and nutrition, and their ability to delignify woody tissues (Leonowicz *et al.* 2001). In addition to their biological functions, laccases are increasingly being investigated for a variety of practical applications including decolorizing and detoxifying effluents, drug analysis, textile dye bleaching, synthesis of polymers and bioremediation (Mayer & Staples 2002). Each application requires unique properties with respect to specificity, stability, temperature and pH dependence. Therefore, screening of microorganisms with laccase activities could facilitate the discovery of novel enzyme for different purposes.

In many fungal species the presence of both constitutive and inducible laccases have been reported and it is present in multiple isoforms with different properties (Leonowicz *et al.* 2001, Mayer & Staples 2002). Although there have been many papers dealing with laccase-producing fungus such as *Trametes versicolor, Phanerochaete chrysosporium*, *Ceriporiopsis subvermispora* and *Agaricus bisporus* (Leonowicz *et al.* 2001), only limited research has been directed towards the laccase produced by members of the genus *Pycnoporus* (Eggert *et al.* 1996, Pointing *et al.* 2000, Vikineswary *et al.* 2006). In the present work we investigated the laccase activities of *Py. sanguineus* and described some of the biochemical properties of two isoforms of laccase from the culture broth.

Materials and methods

Organism and culture conditions

Pycnoporus sanguineus CCT-4518 was obtained from Fundação André Tosello, Campinas, São Paulo, Brazil. *Py. sanguineus* conidia (1.0×10^7 conidia ml^{-1}) were inoculated in liquid medium containing 12.5 g malt extract/l, 0.005 g

$CuSO_4$/l, 1 gl Tween 80/l, and 0.05 g 2,5-xylidine l^{-1}, and incubated at 28 °C with shaking (140 rpm). Fungal biomass was determined at specific time intervals by vacuum filtering mycelia through filter paper, washed with distilled water and dried to constant weight at 80 °C. Laccase activity was monitored by removing 5 ml culture filtrate at intervals.

Laccase assay

Laccase activity was determined at 30 °C using 0.1 mM syringaldazine ($\varepsilon_m = 6.5 \times 10^4$ M^{-1} cm^{-1}) in reaction mixture (1 ml) containing 50 mM citrate/phosphate buffer (pH 4.8 and 4.2), and 60 μl of the culture supernatant for 5 min. One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 μM syringaldazine under standard assay conditions. Assays were carried out in triplicate for each dilution and at least on two different dilutions. Standard deviation did not exceed 10% of the average values. Protein concentration was determined by the method of Bradford with BSA as standard.

Chromatography method

Enzyme solution was fractionated using a phenyl-Sepharose High Performance column (1×17 cm) equilibrated with 50 mM acetate buffer, pH 5.0, with 2 M ammonium sulphate. Laccase isoforms were eluted with a linear gradient of 2–0 M ammonium sulphate, at 2 ml min^{-1}. Fractions (1 ml) were collected and the protein concentration (280 nm) and enzyme activity were determined.

Electrophoresis and enzymatic activities in gels

After SDS-PAGE (12% w/v), gels were washed three times with 50 mM sodium acetate (pH 4.5) for 45 min, and then incubated at 28 °C for 10 min in a solution containing 0.5 mM ABTS. Protein bands exhibiting laccase activity stained green with ABTS (Gonçalves & Steiner 1996).

Results and discussion

Enzyme production

Laccases is one of the important enzymes produced by fungi that have various biotechnology applications (Mayer & Staples 2002). Among white-rot fungi, *Py. sanguineus* produces laccase on solid substrate fermentation (Vikineswary *et al.* 2006) and submerged liquid culture (Pointing *et al.* 2000). This present work focused on the characterization of two laccases from culture supernatant of *Py. sanguineus* induced by 2,5-xylidine, a commonly used inducer for fungal secretion of laccase (Eggert *et al.* 1996, Périe *et al.* 1998, Jung *et al.* 2002). Laccase activity in the extracellular fluid of *Py. sanguineus* cultures supplemented with 0.05 g 2,5-xylidine/l was enhanced 14-fold (Figure 1). A similar effect has been demonstrated in *Py. cinnabarinus* in which 2,5-xylidine enhanced laccase activity by about 9-fold, compared with cultures without inducer (Eggert *et al.* 1996). Laccase production coincided with the synthesis of an orange pigment by the fungus under induced culture.

Enzyme characterization

The enzyme was purified as shown in Figure 2. Two laccases were seen: Lac I (major fraction) and Lac II (minor fraction). SDS-PAGE analysis of these fractions confirmed them to be laccases (Figure 3) with estimated Mr values of 80 and 68 kDa, respectively. The molecular masses of these laccases are consistent with the molecular masses of most other fungal laccases, which are between 60 and 80 kDa (Mayer & Staples 2002, Thurston 1994). Many laccases are produced as multiple isoforms, e.g. *Trametes villosa* produces at least three isoforms and *Pleurotus ostreatus* four

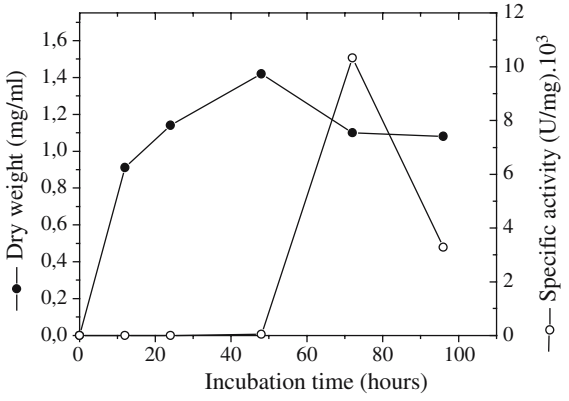

Fig. 1. Time course of laccase production and biomass after growth of *Py. sanguineus* in liquid cultures containing 0.05 g 2,5-xylidine/l as inducer.

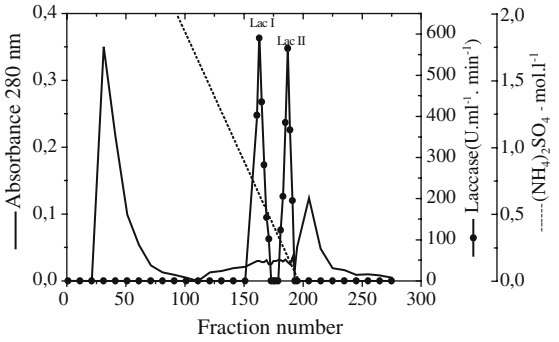

Fig. 2. Elution profile of laccases from a crude extract of *Py. sanguineus* on a column of phenyl-Sepharose (1×17 cm) and equilibrated with 50 mM acetate buffer, pH 5.0, with 2 M ammonium sulphate. Laccase isoforms were eluted with a linear gradient of 2–0 M ammonium sulphate, at 2 ml min^{-1}.

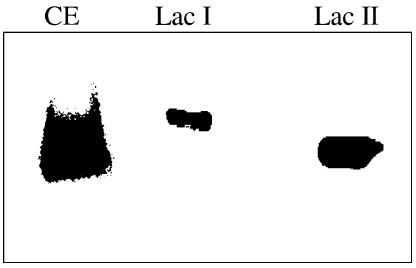

Fig. 3. Activity staining of the isoforms laccase. After SDS-PAGE the enzyme was renatured for washing of gels with 50 mM sodium acetate buffer (pH 4.5) and stained with ABTS. The amounts of protein loaded were 0.3 μg crude extract (CE), 0.5 μg (Lac I) and 0.3 μg (Lac II).

isoforms (Leonowicz *et al.* 2001). This heterogeneity could be due to differences in the extent of glycosylation as described by laccase B from *Trametes sp*. AH28-2 (Xiao *et al.* 2004).

Optimum pHs of Lac I and Lac II were 4.8 and 4.2, respectively (Table 1). Optimum temperatures of Lac I and Lac II were 30 and 50 °C, respectively (Table 1). Lac I was less stable since it presented a fast decline in its activity after incubation at 50 °C, in pH 4.8 for 120 min of pre-incubation. However, Lac II maintained 100% activity at 50 °C even after 120 min. Some fungal laccases are thermostable, although the most white-rot fungi laccases are not active above 50 °C (Cambria *et al.* 2000, Das *et al.* 2001, Hublick & Shinner 2000).

Syringaldazine, which is considered a specific substrate for laccase (Harkin & Obst 1973), exhibited a K_m of 10 μM and 8 μM, for Lac I and Lac II, respectively. In keeping with the general properties of fungal laccases, both enzymes were strongly inhibited by 10 μM sodium azide (Table 1), a common inhibitor of metalloenzymes (Sugumaran 1995).

Table 1. Biochemical properties of the laccases isoforms from *Pycnoporus sanguineus*.

Biochemical properties	Laccase I	Laccase II
Molecular weight	80 kDa	68 kDa
pH optimum	4.8	4.2
Temp. optimum (°C) 5 min	30	50
Temperature stability (50 °C)		
pH 4.8/60 min	71%	ND
pH 4.8/120 min	0	ND
pH 4.2/60 min	ND	100%
pH 4.2/120 min	ND	100%
K_m (μM)	10	8
V_{max} (U ml^{-1} min^{-1})	151	195
Inhibition by NaN_3 (10 μM)	85%	77%

ND: Not Determined. The molecular weights of the enzymes were determined by gel filtration on Sephacryl S-200. The effect of pH on the enzyme activity was determined by varying the pH of the reaction mixtures using citrate/phosphate buffer (pH 2.6–7.0). The effect of temperature on the enzymatic activity was determined at the pH optimum, in the range of 20–70 °C. The effect of temperature on the enzyme stability was analyzed by previously incubating the enzyme at 50 °C for 60 and 120 min. Michaelis-Menten constant (K_m) was determined by non-linear-regression analysis of data obtained by measuring the rate of oxidation of syringaldazine (from 5 to 60 μM). The inhibition of the enzymes activities by NaN_3 was determined through previous incubation of the enzyme with 10 μM during 5 min. Results are means values of three replicates.

Acknowledgments

This work was supported by Grants from CNPq, FUNAPE/UFG and International Foundation for Science (IFS – W/3433-1). TAG was supported by CAPES/Brazil.

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