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SHORT COMMUNICATION

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Properties of a laccase produced by *Phanerochaete flavido-alba* induced by vanillin

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Abstract *Phanerochaete flavido-alba* is able to remove simple and polymeric phenols from the recalcitrant wastes of the olive oil industry, in a process in which a laccase is involved. This report describes the characterization of a laccase produced by *P. flavido-alba* and induced by vanillin. Although the amino acid composition of the purified enzyme is typical for laccases, other molecular characteristics show that it is quite different from fungal laccases. The purified laccase oxidized preferably *o*- and *p*-biphenols.

Keywords Laccase · *Phanerochaete* · *Phanerochaete flavido-alba*

Introduction

The production of extracellular phenoloxidases is a characteristic of the ligninolytic metabolism of the white-rot fungi, the most efficient lignin degraders. Lignin peroxidase, manganese-dependent peroxidase (MnP) and laccases are the main phenoloxidases involved in lignin degradation. Laccases (benzenediol oxygen oxidoreductases, EC 1.10.3.2) are glycoproteins characterized by their copper content and their oxidative activity on phenols, aromatic amines and ascorbate. *Phanerochaete chrysosporium*, the best known ligninolytic fungus, has been considered for many years as a striking exception because no laccase was detected in cultures. Only two papers (Dittmer et al. 1997; Srinavasan et al. 1995) deal with the properties of *P. chrysosporium* laccases. Little information about laccases in other *Phanerochaete* species has been published (Bogan and Lamar 1996). *P. flavido-alba* laccase is involved in the detoxification of effluents from olive oil production (Pérez et al. 1998). These wastes are highly recalcitrant, mainly because of the phenolic content (Martínez et al. 1998). Detoxification by *P. flavido-alba* is accompanied by an induction of laccase. In this report, we describe *P. flavido-alba* laccase induction by several aromatic compounds. Since the compound yielding higher laccase induction was vanillin, the chromatographic, electrophoretic and molecular properties of the *P. flavido-alba* laccase induced by vanillin were determined.

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Materials and methods

Microorganism and culture conditions

P. flavido-alba FPL 106507 was cultured in 250-ml or 1,000-ml Erlenmeyer flasks containing 25 ml or 300 ml of basal culture (BL) medium at pH 4.5 (Pérez et al. 1996). Static cultures were incubated for 7 days or 11 days at 30 °C. The effect of aromatic compounds on laccase production was determined in BL medium containing 22.4 µM $CuSO_4$. Fourteen aromatic compounds were assayed: guaiacol, veratryl alcohol, *p*-hydroxybenzaldehyde, vanillin and the acids 3,4,5-trimethoxybenzoic, benzoic, veratric, gallic, protochatechuic, *p*-hydroxybenzoic, vanillic, caffeic, ferulic and *p*-cumaric. Each substance, dissolved in dimethylformamide:water (3:7, v/v) was added (at 1 mM) to 2-day-old cultures. After incubation for additional 5 days, growth and extracellular laccase activity were determined.

Enzyme activity assays

Extracellular laccase and MnP activities were determined at 30 °C, as described by Pérez et al. (1996). The enzymatic activity was expressed as nanokatals per milliliter, nanokatals per milligram of dried mycelium and nanokatals per milligram of purified enzyme. Dry weight was measured by filtering triplicate cultures and drying the mycelium for 24 h at 105°C.

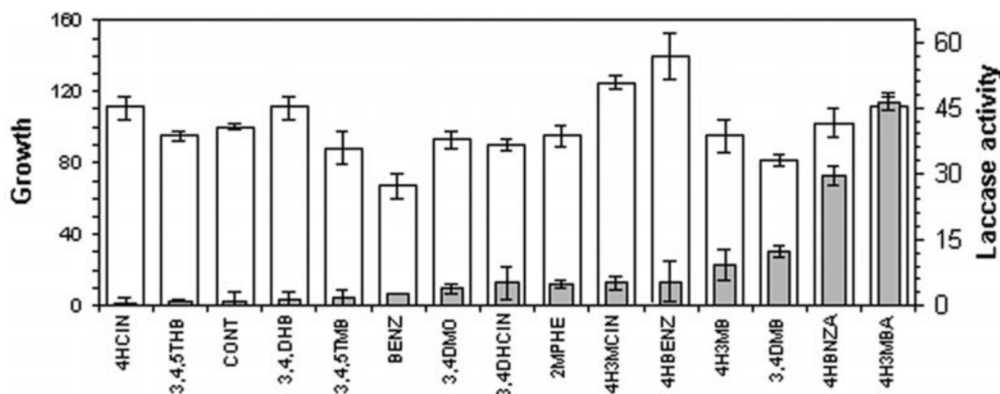

Fig. 1 Effect of aromatic compounds on growth and laccase activity of *P. flavido-alba*. Growth (*white columns*) is expressed as the ratio (%) between the dry weight of aromatic-added cultures and that of control cultures. Specific laccase activity (*gray columns*) is expressed as the ratio between the activity in the aromatic-added cultures and that in control cultures. *4HCIN* *p*-Coumaric acid, *3,4,5THB* gallic acid, *CONT* Control, *3,4DHB* protochatechuic acid, *3,4,5TMB* 3,4,5-trimethoxybenzoic acid, *BENZ* benzoic acid, *3,4DMO* veratryl alcohol, *3,4DHCIN* caffeic acid, *2MPHE* guaiacol, *4H3MCIN* ferulic acid, *4HBENZ* *p*-hydroxybenzoic acid, *4H3MB* vanillic acid, *3,4DMB* veratric acid, *4HBNZA* *p*-hydroxybenzaldehyde, *4H3MBA* vanillin

Laccase purification and characterization

The constitutive *P. flavido-alba* laccase and the laccase induced by vanillin were purified from extracellular fluids from 11-day-old cultures in BL medium or in BL plus 1 mM vanillin (BL-Va), according to Pérez et al. (1996). Protein content was determined by a protein assay (Bio-Rad, Richmond, Calif.).

SDS-PAGE was done as described elsewhere. Gels were stained for proteins (with Serva violet, from Serva Fine Biochemicals) and for glycoproteins (with thymol-sulfuric acid; Gerad 1990). The carbohydrate content of laccase was determined by SDS-PAGE before and after deglycosylation with endo-β-*N*-acetylglucosaminidase H (Boehringer Mannheim). Analytical isoelectric focusing at pH 3–6 was done, as described by Pérez et al. (1996). Laccase activity was detected by immersing the gels in 10 mM 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS) in 0.1 M sodium acetate, pH 5.0.

The N-terminal sequence of laccase was determined by automated Edman degradation of 5 μg of protein in an Applied Biosystems 477A pulsed-liquid protein sequencer with a model 120A phenylthiohydantoin analyzer. The amino acid composition of the purified laccase was determined after acid hydrolysis of 200 μg of protein with HCl and analysis by HPLC (Biochrom-20, Pharmacia) and ninhydrin detection.

The rate of aromatic substrate oxidation was determined by spectrophotometry, using published molar extinction coefficients (ε). Apparent K_m and V_{max} values were determined (in the range 0.57–11.50 mM) for methylhydroquinone (ε_{250} =21,112), 2,6-dimethoxyphenol (DMP; ε_{468} =27,500), catechol (ε_{392} =1,456) and ABTS (ε_{436} =29,300) oxidation in 0.25 M glycine buffer (pH 3.0) at 30 °C. All kinetic studies were performed at least three times and the kinetic data were fitted to a rectangular hyperbola by computer.

The effect of inhibitors (sodium azide and sodium diethyldithiocarbamate, DEDTCS) was determined by incubating enzyme with various concentrations (1–10 mM) of inhibitor and measuring ABTS oxidation after 15 min. The inhibition was expressed as the IC_{50}.

Results and discussion

We reported that an induced laccase plays an important role in the decolorization and detoxification of olive oil mills by *P. flavido-alba* (Pérez et al. 1998). We also demonstrated that olive oil wastewater phenols are responsible for *P. flavido-alba* laccase induction during the detoxification of this wastewater (Ruiz et al. 2002). Here, we assayed several inducers and studied the enzymatic and molecular properties of the laccase induced by vanillin. Vanillin and *p*-hydroxybenzaldehyde yielded cultures containing high laccase activity (Fig. 1). *P. flavido-alba* laccase production was not affected by other laccase inducers, such as veratryl alcohol, guaiacol, or ferulic acid.

Fig. 2A–C Electrophoresis of *P. flavido-alba* purified laccase from cultures on basal culture medium plus 1 mM vanillin, after anion-exchange and gel filtration chromatography. *Lane S* Standard protein markers. **A** SDS-PAGE of purified laccase stained with Coomassie blue (*lane 1*) and stained for glycoproteins (*lane 2*). **B** Coomassie blue-stained SDS-PAGE gel of purified laccase (*lane 1*) and deglycosylated protein (*lane 2*). **C** Isoelectric focusing of purified laccase stained for proteins (*lane 1*) and for laccase activity (*lane 2*)

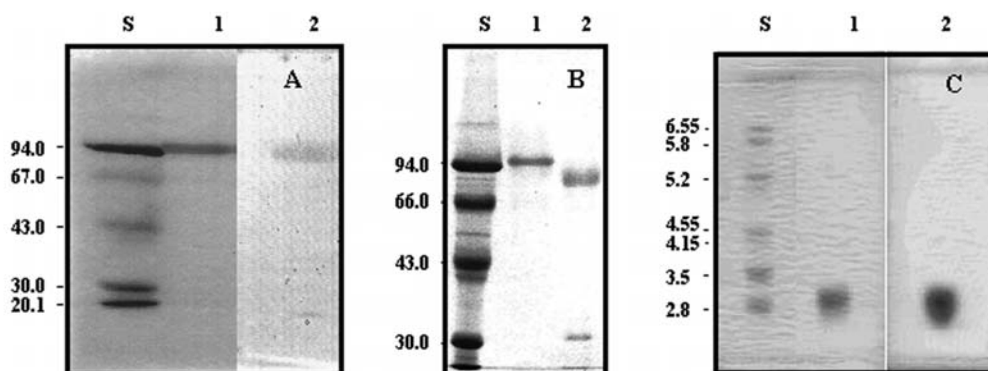

Table 1 Comparison of N-terminal amino acid sequences of *P. flavido-alba* laccase and other basidiomycete laccases (*Lac*). Numbers in parentheses identify the relevant reference: *(1)* Yaver et al. (1996), *(2)* Ong et al. (1997), *(3)* Mansur et al. (1997), *(4)* Saloheimo et al. (1991), *(5)* Coll et al. (1993), *(6)* Giardina et al. (1995), *(7)* Kojima et al. (1990), *(8)* Yaver et al. (1999), *(9)* Salas et al. (1995), *(10)* Fukushima and Kirk (1995)

Laccase	Amino acid sequence alignment
P. flavido-alba induced Lac	D T V S L P S S S D I I L N G L Q G Q A P Q T R N Y D F V
P. flavido-alba constitutive Lac	D T V S L P S S S D I I L N
Trametes versicolor Lac 1 (1)	A I G P V A S L V V A N A P – V S P D G F
T. versicolor Lac 4 (2)	V F G A I G P V T D L T I S N A D – V T P
T. villosa Lac 1 (1)	A G I G P V A D L T I T N A A – V S P D G
T. villosa Lac 2 (3)	A I G P V A S L V V A N A P – V S P D G F
T villosa Lac 3 (1)	A S I G P V A E L D I V N K V – I A P D G
Phlebia radiata Lac 1 (4)	A S I G P V T D F H I V N A A – V S P D G
Basidiomycete PM1 (5)	A S I G P V A D L T I S N G A – V S P D G
Basidiomycete CECT 20197 (3)	L N T F A A G P V T D L T I S N A N – V
Pleurotus ostreatus Lac 1 (6)	T H A A I G P T G D M Y I V N E D – V S P
Coriolus hirsutus Lac 1 (7)	A A I G P T A D L T I S N A E – V S P D G
Coprinus cinereus Lac 1 (8)	V N S V D T M T L T N A N – V S P D G F T
C. cinereus Lac 2 (8)	G P S T N L V V A N K V – I A P D G F S R
C. cinereus Lac 3 (8)	V G N L V I A N A N – V S P D G F V R S A
Ceriporiopsis subvermispora pI 3, 60 (9)	A I G P V T D I E I T D A F – V S P P H P
C. subvermispora L1 (10)	A I G P V T D L E I T D A F – V S P D G P

Constitutive and induced *P. flavido-alba* laccases seemed to be the same, because both showed the same molecular mass, pI and N-terminal sequence. The induced laccase purified from *P. flavido-alba* cultures in BL-Va medium was identified as a glycoprotein (Fig. 2A). After deglycosylation, its molecular mass decreased from 98.7 kDa to 81.3 kDa for the deglycosylated protein (Fig. 2B). The glycoprotein contains more than one high mannose or hybrid type asparagine-bound glycosydic chain (according to the Boehringer deglycosylation kit protocol). The unusually high molecular mass of the *P. flavido-alba* constitutive and induced laccases could be due in part to the carbohydrate content (17.7%), which is higher than the common white-rot fungal laccases (Farnet et al. 2000; Muñoz et al. 1997). However, white-rot fungal laccases containing hydrocarbons in excess of 20% have been described (Salas et al. 1995). Zymograms of the glycosylated enzyme show a diffuse laccase band at pI 2.8–3.5. (Fig. 2C). The purified laccase shows an absorption spectrum typical of these copper-containing proteins. The absorbance ratio A_{280}/A_{610} was 38.5.

The amino acid composition of the purified protein (%mol) was as follows: Asp 13.14, Thr 11.13, Ser 8.62, Glu 8.71, Gly 11.02, Ala 6.75, Cys 0.60, Val 5.93, Met 1.55, Ile 3.92, Leu 7.55, Tyr 3.21, Phe 4.04, Hys 3.03, Lys 0.81, Arg 2.72 and Pro 7.23. When this amino acid composition was compared (using the ExPASy server) with proteins deposited in data banks, it showed the greatest degree of similarity with fungal laccases. However, the N-terminal sequence did not exhibit similarity to any other laccases described (Table 1). The N-terminal amino acid sequences of the *Coriolus hirsutus* and *Phellinus ribis* laccases had low similarity with other basidiomycete laccases (Min et al. 2001; Shin and Lee 2000). The constitutive and *p*-hydroxybenzoic induced laccases from *Marasmius quercophilus* had different amino acid compositions and enzymatic properties (Farnet et al. 2000).

Table 2 Kinetic parameters of *P. flavido-alba* laccase for oxidation of various substrates with 74 µmol min^{-1} mg^{-1} purified enzyme. All values are the means of triplicate measurements. *ABTS* 2,2′-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic) acid

Substrate	K_m (mM)	V_{max} (µmol min^{-1} mg^{-1})
Methylhydroquinone	0.67	280
2,6-dimethoxyphenol	0.76	290
ABTS	1.19	727
Catechol	1.66	150

The substrate range of *P. flavido-alba* laccase induced by vanillin, at the optimum pH for ABTS oxidation, was common to other laccases. It oxidized *o*- and *p*-diphenols (catechol, hydroquinone), but did not oxidize tyrosine. Higher K_m values were observed, especially for ABTS, compared with other laccases (Table 2; Saparrat et al. 2002). Vanillin-induced laccase showed a major affinity for methylhydroquinone and DMP. As with *Pleurotus eryngii* laccase (Guillén et al. 2000), dimethoxy derivatives (DMPs) were better substrates than monomethoxylated phenols (*p*-methoxyphenol). Phenoxy-acids were not oxidized by *P. flavido-alba* laccase.

Laccase activity was inhibited by Cu-chelating agents. The enzyme was more sensitive to DEDTCS (IC_{50}= 0.98 mM) than to sodium azide inhibition (IC_{50}=5.1 mM). This difference must be interpreted with caution, because sulfhydryl-chelating agents can underestimate laccase activity measured as ABTS oxidation (Johannes and Malcherczyk 2000).

The description of newly discovered activities of ligninolytic enzymes, such as Mn (II) oxidation by *Trametes versicolor* laccase (Hofer and Schlosser 1999), adds more pieces to the old puzzle of enzymatic composition and activities in the ligninolytic enzymatic arrangement of ba-

sidiomycetes. In this context, the special molecular enzymatic characteristics of *P. flavido-alba* laccase increase our knowledge of fungal laccases in general and of the controversial *Phanerochaete* spp laccases in particular. However, further studies must be carried out in order to clarify the importance of laccase in the *Phanerochaete* spp ligninolytic system.

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