

Biochemical and molecular characterization of *Corioloopsis rigida* laccases involved in transformation of the solid waste from olive oil production

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Abstract Two laccase isoenzymes were purified and characterized from the basidiomycete *Corioloopsis rigida* during transformation of the water-soluble fraction of “alpeorujo” (WSFA), a solid residue derived from the olive oil production containing high levels of toxic compounds. Zymogram assays of laccases secreted by the fungus growing on WSFA and WSFA supplemented with glucose showed two bands with isoelectric points of 3.3 and 3.4. The kinetic studies of the two purified isoenzymes showed similar affinity on 2,6-dimethoxyphenol and 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid), used as phenolic and non-phenolic model substrate, respectively. The molecular mass of both proteins was 66 kDa with 9% N-linked carbohydrate. Physico-chemical properties of the purified laccases from media containing WSFA were similar to those obtained from medium with glucose as the main carbon source. In-vitro studies performed with the purified laccases revealed a 42% phenol reduction of WSFA, as well

as changes in the molecular mass distribution. These findings indicate that these laccases are involved in the process of transformation, via polymerization by the oxidation of phenolic compounds present in WSFA. A single laccase gene, containing an open reading frame of 1,488 bp, was obtained in PCR amplifications performed with cDNA extracted from mycelia grown on WSFA. The product of the gene shares 90% identity (95% similarity) with a laccase from *Trametes trogii* and 89% identity (95% similarity) with a laccase from *Corioloopsis gallica*. This is the first report on purification and molecular characterization of laccases directly involved in the transformation of olive oil residues.

Keywords Olive-mill waste · Basidiomycetes · Phenol · Gene sequence

Introduction

The commercial olive oil production by a two-phase extraction system generates a semi-solid organic waste, named “alpeorujo”. This by-product is further dried and extracted with solvents to obtain an extra yield of oil and a dry olive-mill residue (Vlyssides et al. 1998), which has a great potential as fertilizer or amendment due to high content in both organic and inorganic nutrients (Sampedro et al. 2007). However, the main technical constraint to the biological upgrading of this residue is due to the presence of toxic compounds, including a water-soluble phenolic fraction, which has been shown to have phytotoxic (Sampedro et al. 2005; Bonanomi et al. 2006) and antimicrobial properties (Kotsou et al. 2004; Saparrat et al. 2010). Although many approaches have been explored to

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detoxify both solid and liquid residues from the olive oil industry, including thermal processes, electrolysis, ozonation, and evaporation, among others, bioremediation has gained increasing importance in recent years. The treatment with fungi such as *Pleurotus* spp., *Chalara paradoxa*, and *Lentinula edodes* has been shown to be useful in the decolorization and detoxification of olive oil mill wastewaters (Robles et al. 2000; Tsioulpas et al. 2002; Dias et al. 2004).

Corioloopsis rigida is a white-rot fungus which has been extensively studied for its ability to degrade lignin, polycyclic aromatic hydrocarbons, and dyes (Capelari and Zadrazil 1997; Gómez et al. 2006; Sánchez-López et al. 2008). In addition, this fungus is able to detoxify the water-soluble fraction of “alpeorujo” (WSFA), which contains most of the toxic phenolic compounds from the dry residue, and thus reducing its phyto- and microtoxicity through phenol polymerization (Sampedro et al. 2004; Aranda et al. 2007; Saparrat et al. 2010). Although the mechanisms of detoxification of olive oil by-products and effluents remained controversial (de la Rubia et al. 2008), it has been recently demonstrated that at least in the case of *C. rigida*, the action of its extracellular laccases on phenol content is involved in the reduction of the residue toxicity as revealed against *Azospirillum brasiliense*, a N₂-fixing soil rhizobacterium which promotes plant growth (Saparrat et al. 2010).

Laccases (*p*-diphenol: oxygen oxidoreductase; EC 1.10.3.2) are phenol-oxidases which catalyze the oxidation of a great variety of phenolic compounds and aromatic amines using molecular oxygen as electron acceptor (Baldrian 2006), and due to their properties, they have been widely used for industrial applications, including pulp bleaching in paper industry, dye decolorization, and detoxification of environmental pollutants (Mayer and Staples 2002; Saparrat et al. 2008). The aim of this work was to understand and identify the mechanisms of transformation of WSFA by *C. rigida*. We have hence purified and characterized two laccase isoenzymes secreted by *C. rigida* grown on WSFA and demonstrated their direct effect in the transformation of “alpeorujo” via polymerization of soluble compounds present in the residue. In addition, a molecular characterization of the gene encoding both isoenzymes is provided.

Methods

Water-soluble fraction of “alpeorujo”

Olive-mill dry residue was collected from an olive oil manufacturer (Aceites Sierra Sur S.L., Granada, Spain), autoclaved, and stored at 4°C until use. WSFA was

obtained by Soxhlet extraction of olive-mill dry residue with water by using a solid/liquid ratio of 1:8 (*w v*⁻¹) for 16 h, and then filtered by vacuum through a 0.22-μm membrane and stored at -20°C until use.

C. rigida cultures

A dikaryon of *C. rigida* strain LPSC No. 232, from the culture collection of the La Plata Spegazzini Institute (= Spanish Type Culture Collection, CECT, n° 20449), was grown on peptone–yeast extract medium (Saparrat et al. 2002), where glucose was substituted by WSFA as the main carbon source at different concentrations (25%, 50%, and 100%) and pH adjusted to 5.0 with HCl. *C. rigida* was also grown on this peptone–yeast extract medium containing both 50% WSFA and glucose (either 5 or 10 g L⁻¹) in order to increase the enzyme production for further purification studies, as well as on the basal medium, with glucose–peptone–yeast extract, supplemented with 150 μM CuSO₄ (Saparrat et al. 2002), in order to compare the zymogram profiles of laccases secreted in this medium with those produced on cultures with WSFA. In all cases, a mycelial suspension (1%, *v v*⁻¹) was inoculated in 1 L Erlenmeyer flasks containing 200 mL of each medium. The mycelial suspensions were obtained from homogenized pellets from 5-day-old shaken cultures, grown on the basal medium with glucose and 150 μM CuSO₄ (Saparrat et al. 2002). Three replicate cultures of each media were grown at 150 rpm and 28±1.5°C in the darkness for 57 days in a chamber with controlled humidity to minimize the evaporation.

Analytical procedures

Samples (2 mL) were taken periodically from the cultures, and the supernatants, separated from mycelia by centrifugation at 8,000×g for 10 min, were analyzed for different parameters. Phenol content was determined by the Folin-Ciocalteu reagent (Singleton and Rossi 1965), using tannic acid as the standard. Laccase activity was assayed by the oxidation of 5 mM 2,6-dimethoxyphenol (DMP) to coerulignone ($\epsilon_{469}=27,500 \text{ M}^{-1} \text{ cm}^{-1}$) in 100 mM sodium acetate buffer (pH 5.0). Peroxidase (EC 1.11.1.7) activity was assayed as laccase activity in the presence of 0.1 mM H₂O₂ (Saparrat and Guillén 2005). Manganese peroxidase (EC 1.11.1.13) activity was estimated by measuring the formation of Mn⁺³-tartrate complex ($\epsilon_{238}=6,500 \text{ M}^{-1} \text{ cm}^{-1}$) during the oxidation of 0.1 mM MnSO₄ in 100 mM sodium tartrate buffer (pH 5.0) and 0.1 mM H₂O₂ (Saparrat and Guillén 2005). International enzymatic units (micromoles per min) were used. Proteins were determined according to Bradford method, using bovine albumin as standard and Bio-Rad kit assay.

Laccase purification

The purification process was carried out with extracellular crude extracts obtained from 57-day-old cultures growing on 50% WSFA and 10 g L⁻¹ glucose, which showed the highest laccase activity levels. The liquid culture was separated from mycelia by centrifugation at 20,000×*g* for 20 min, and the supernatant was collected and concentrated by ultrafiltration (Pall Filtron, 3-kDa cutoff membrane) and then dialyzed against 10 mM sodium acetate (pH 5.0).

To design the purification process, the pH stability of laccase activity was studied in 100 mM borate–citrate–phosphate buffer (pH 3 to 7) at room temperature for 72 h. Crude enzyme preparation was incubated in the same buffer (pH 7.0) at 4°C and room temperature for 72 h to analyze thermal stability.

The crude enzyme preparation was applied to a HiTrap-Q (Amersham Biosciences) column equilibrated with 10 mM sodium acetate buffer pH 5.0 at a flow rate of 1.5 mL min⁻¹. Retained proteins were eluted for 160 min with a NaCl gradient from 0 to 1 M NaCl and maintaining this salt concentration for 10 min to elute proteins and pigments retained. Fractions of 4.5 mL were collected in tubes containing 0.45 mL of 500 mM phosphate buffer (pH 7.0). Fractions with the laccase activity were pooled and concentrated (Filtron Microsep; 3-kDa cutoff), and samples of 1 mL were applied to a Superdex 200 (Amersham Pharmacia Biotech HR 16/60) column equilibrated with 50 mM phosphate buffer (pH 7.0) containing 150 mM NaCl at a flow rate of 0.4 mL min⁻¹. The laccase peak was pooled, concentrated (Filtron Microsep, 3-kDa cutoff), and dialyzed against 10 mM sodium acetate (pH 5.0) by a PD-10 desalting column (Amersham Biosciences). Then, 1 mL samples were applied to a Mono-Q anion-exchange column (Pharmacia HR 5/5) equilibrated with the same buffer. Both laccase isoenzymes were eluted with a linear NaCl gradient from 0 to 250 mM for 42 min and from 250 mM to 1 M for 5 min at a flow rate of 0.8 mL min⁻¹. Fractions of 2 mL were collected, and laccase peaks were pooled, concentrated, and stored with 10% (*w v*⁻¹) glycerol at -4°C.

The laccase isoenzymes secreted in basal medium with glucose and copper were also purified but using the previously reported protocol (Saparrat et al. 2002).

Enzyme characterization

A preliminary characterization of laccase was performed by isoelectric focusing using the extracellular crude extracts from cultures grown on WSFA and WSFA supplemented with glucose. The isoelectric point (pI) was determined by zymograms performed on 5% polyacrylamide gels with a thickness of 1 mm by using a pH range from 3 to 10 (Bio-Rad

Ampholine). The sample (20 µl) contained 5–10 mU of laccase. The anode and cathode solutions were 1 M phosphoric acid and 1 M sodium hydroxide, respectively. The pH gradient was measured on the gel by means of a contact electrode. Protein bands with laccase activity were detected by using 5 mM DMP in 100 mM sodium acetate buffer (pH 5.0) after the gels were washed for 10 min with the same buffer.

The molecular mass of isoenzymes was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion chromatography. SDS-PAGE was performed with 7.5% polyacrylamide gels by using low- and high-molecular-mass standards (Bio-Rad). Size exclusion chromatography was carried out on Superdex 200 column as described above. The column was calibrated with blue dextran (2,000 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). The N-glycan content of purified laccases was determined by the difference in molecular mass (estimated by SDS-PAGE) found before and after treatment of laccase isoenzymes with endo-*N*-acetylglucosaminidase.

The kinetic constant K_m was calculated for LacI and LacII from cultures grown on WSFA and glucose, as well as for laccases obtained from the basal medium with glucose and copper, using DMP as model phenolic substrate and 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as non-phenolic substrate.

WSFA treatment with laccase

Enzymatic treatment was carried out for 24 h at room temperature on 40 µL of WSFA diluted 1:10 with 500 mM sodium phosphate buffer at pH 7.0, since although optimum pH for both LacI and LacII was 2.0, highest enzyme stability in presence of WSFA after 24 h was achieved at pH 7.0 (95% of residual activity). The laccase used (1 U mL⁻¹) was a mixture of LacI and LacII (obtained from WSFA media, after the size-exclusion chromatography). At the end of incubation, phenolic content on liquid fraction was analyzed. Changes in the molecular mass distribution of the WSFA fractions after enzymatic treatment were also analyzed as described by Jaouani et al. (2005) through size-exclusion chromatography on Sephadex G-100 column (Pharmacia, 1×48 cm) equilibrated with 50 mM NaOH and 25 mM LiCl₂, at a flow rate of 0.4 mL min⁻¹, according to Sarkanen et al. (1982). Blue dextran and syringic acid were used as low- and high-molecular mass standards, respectively.

Preparation of genomic DNA, total RNA, and reverse transcription

The mycelium was collected from 57-day-old cultures with 50% WSFA and 10 g L⁻¹ glucose, corresponding to the

maxima levels of laccase activity, separated from the culture liquid by centrifugation at 20,000×g for 20 min, washed with water treated with diethylpyrocarbonate to inactivate RNAases, and kept at −80°C for RNA extraction. Likewise, 5-day-old mycelium, grown in basal medium with glucose medium, was used for genomic DNA extraction as described below.

Total RNA was isolated using Ultraspec RNA (Biotech Laboratories, Inc.) and treated with DNase I using the “Deoxyribonuclease I, Amplification Grade” (Invitrogen, UK), to remove the chromosomal DNA contamination from the samples. First-strand cDNA was synthesized using the “GeneAmp Gold RNA PCR Reagent Kit” (Applied Biosystems, USA). This cDNA was used as template in polymerase chain reactions (PCR) described below. Genomic DNA was obtained using Genomix DNA extraction kit (Talent, Italy) according to manufacturer's instructions.

PCR amplification and cloning of the laccase gene fragment

The cDNA obtained as described above, was used as template for PCR amplification. Degenerate primers CR1 (5' GCNATHGGNCCNAARGC 3') designed on the basis of the N-terminal sequence of laccase from *C. rigida* purified from basal medium with glucose and copper (Saparrat et al. 2002), and P_{Cu4} (5' TGRAARTCDATRTGRCARTG 3'), based on the conserved sequence of the copper-binding region IV (HCHIDFH) in fungal laccases (Hong et al. 2007), were used to amplify laccase genes from *C. rigida* growing on WSFA. Amplified fragments were inserted into pGEM-T easy cloning vector (Promega). After transformation of the recombinant vectors into the *Escherichia coli* DH5α strain, the clones containing the inserted fragments were isolated and verified by DNA sequencing using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and the automated ABI Prism 3730 DNA sequencer (Applied Biosystems). The sequence was compared by BLAST search in GenBank (Altschul et al. 1997).

Amplification of the flanking sequences of the laccase gene

In order to obtain the flanking sequence of the laccase gene corresponding to the C-terminus of the protein, primer CR2 (5' AACGACGCCATAAGCCCAAAC 3') was designed based on 3' extreme from fungal laccase sequences with the highest homology (see “Results” section), and primer CR3 (5' GCCATCGGGCCCAAGG 3') was designed on the 5' extreme from the laccase gene fragment obtained as described above.

PCR reaction was carried out with these two primers using 0.2 μg of either cDNA or DNA as template. Cycling parameters were 95°C for 3 min followed by 35 cycles of

94°C for 1 min, 52°C for 40 s, and 72°C for 1 min and the final extension at 72°C for 10 min.

In-gel trypsin digestion

Digestion of protein bands corresponding to LacI and LacII was performed with trypsin by using the DigestPro MS system (Intavis AG, Germany) following the standard protocol. The elution mixture was then dried down in a speed vacuum and resuspended in 4 μL 30% acetonitrile and 0.1% TFA.

MS and MS/MS analysis were performed with an Autoflex Smartbeam TOF/TOF (Bruker Daltonics, Germany) spectrometer equipped with a LIFT ion selector and a Reflectron ion reflector. Data collection was performed in fully automated, fuzzy-logic mode. Typically, 1,000 scans for peptide mass fingerprint and 200 scans for MS/MS were collected. Automated analysis of mass data was performed using flexAnalysis 3.0 software (Bruker-Daltonics, Germany). Internal calibration of MALDI-TOF mass spectra was performed using two trypsin autolysis ions with m/z 842.510 and m/z 2,211.105; for MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the m/z 800–3,200 region.

MALDI-MS and MS/MS data were combined through the Biotoools 3.0 software (Bruker-Daltonics, Germany) to search a nrNCBI database using MASCOT software (Matrix Science, UK). The following parameters were used for MASCOT searching: enzyme, trypsin; fixed modifications, carbamidomethyl (C); allow up to 1 missed cleavage; peptide tolerance 20 ppm, MS/MS tolerance 0.5 Da.

Results

Enzyme activity on WSFA

Activities of ligninolytic enzymes including laccase, peroxidase, and manganese peroxidase were assayed in the supernatant of cultures with different WSFA concentrations. Laccase was the single extracellular ligninolytic activity detected at the three concentrations of WSFA tested (25%, 50%, and 100%), reaching the highest activity levels after 21 days of incubation (Fig. 1a). Since no difference was found between 21-day-old cultures containing 100% or 50% WSFA, the effect of glucose (5 and 10 g L⁻¹) was only evaluated on cultures with 50% WSFA (Fig. 1b). The highest activity levels were achieved on 57-day-old cultures in the presence of 10 g L⁻¹ of glucose (1,466 mU mL⁻¹).

A preliminary characterization of an extracellular laccase preparation from cultures of *C. rigida* grown on 50% WSFA, 50% WSFA and glucose (10 g L⁻¹) as well as on

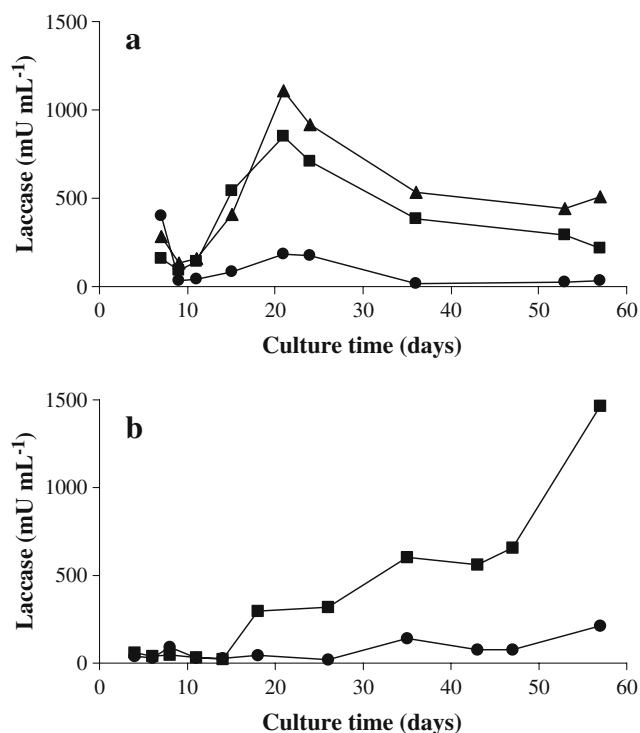


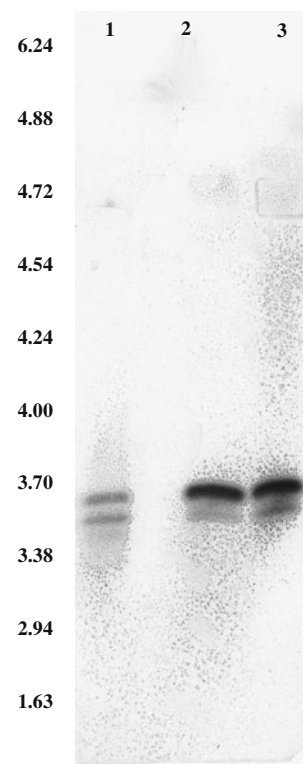
Fig. 1 **a** Laccase activity (mU mL^{-1}) from *C. rigida* cultures grown in basal medium, without glucose, with 25% (filled circle), 50% (filled square), or 100% WSFA (filled triangle). **b** Laccase activity (mU mL^{-1}) of *C. rigida* cultures grown in basal medium with 50% WSFA supplemented with 5 (filled circle) or 10 g L^{-1} of glucose (filled square)

glucose (10 g L^{-1}), and $150 \mu\text{M CuSO}_4$ was carried out to design the purification process. Zymograms revealed that two laccase isoenzymes produced in the medium containing only WSFA were similar to those produced in WSFA supplemented with glucose (Fig. 2). The pI of laccase activity bands were 3.3 and 3.4, similar to those observed in a previous report in cultures of *C. rigida* grown in the basal medium with copper (Saparrat et al. 2002). Thus, the full characterization and purification of laccases was carried out using extracellular crude extracts produced in WSFA medium with 10 g L^{-1} glucose, since it provided slightly higher activity levels and less WSFA concentration, and also to reduce interferences during the purification process associated with waste components. Laccase activity was stable at both 4°C and 25°C , retaining 81% of activity after 72 h in 100 mM borate–citrate–phosphate buffer pH 7.0. A dramatic decrease in laccase activity was observed at acidic pH (88% of lost activity after 72 h incubation at pH 3.0).

Purification of laccase

Table 1 summarizes the purification process of *C. rigida* laccases secreted in 50% WSFA medium supplemented with glucose. During the first chromatography step (Q-Column),

Fig. 2 Zymograms in isoelectrofocusing gels of *C. rigida* laccases secreted in different culture conditions: 50% WSFA (lane 1), 50% WSFA and glucose (lane 2), glucose and $150 \mu\text{M CuSO}_4$ (lane 3). pI values are given to the left



the laccase activity was separated from most of impurities, although color was still present due to the chromophores from WSFA which showed high absorbance at 280 nm (Fig. 3a). A major protein peak with laccase activity was obtained with the size-exclusion chromatography (Superdex 200) (Fig. 3b). The last chromatographic step involving a high-resolution ion-exchange column (Mono-Q) was necessary to resolve two laccase activity peaks: LacI and LacII (Fig. 3c). At the end of the process, LacI and LacII were purified 32.7- and 31.2-fold, respectively. The overall yield of the purification was 2.2% and 2.6% for LacI and LacII, respectively (Table 1).

To compare the catalytic properties of two laccase isoenzymes produced by *C. rigida* on WSFA with those reported previously from a basal medium with copper (Saparrat et al. 2002), they were also purified to homogeneity, obtaining similar efficiency of purification to those reported in the mentioned study.

Enzyme characterization

The molecular masses of both LacI and LacII obtained from cultures with WSFA were 60 kDa, estimated by size-exclusion chromatography, and 66 kDa determined by SDS-PAGE (Fig. 4). The difference between the molecular mass of each laccase and its corresponding deglycosylated form (after SDS-PAGE) revealed that both LacI and LacII are glycoproteins with 9% N-linked carbohydrate (Fig. 4). The same properties were obtained from the isoenzymes

Table 1 Purification of laccase isoenzymes from *C. rigida* grown in the presence of WSFA and glucose

Purification step	Protein (mgL ⁻¹)	Activity (UL ⁻¹)	Specific activity (Umg ⁻¹)	Yield (%)	Purification factor (fold)
Culture liquid	3.600	1,983.0	550.8	100.0	1.0
HiTrap-Q	0.174	405.0	2,327.6	20.4	4.2
Superdex 200	0.007	225.0	32,090.9	11.3	61.9
Mono-Q (LacI)	0.002	43.2	18,000.0	2.2	32.7
Mono-Q (LacII)	0.003	51.6	17,200.0	2.6	31.2
Mono-Q (LacI+LacII)	0.005	94.8	35,200.0	4.8	63.9

purified from basal medium with glucose and copper (Saparrat et al. 2002).

The K_m of both LacI and LacII isoenzymes purified from media supplemented with WSFA and glucose were respec-

tively 14 μM for ABTS and 21.2 and 18.5 μM for DMP. In the case of laccases from the basal medium with glucose and copper, the K_m values of both LacI and LacII were 12 and 11 μM for ABTS, and 15.3 and 17.5 μM for DMP, respectively (Saparrat et al. 2002).

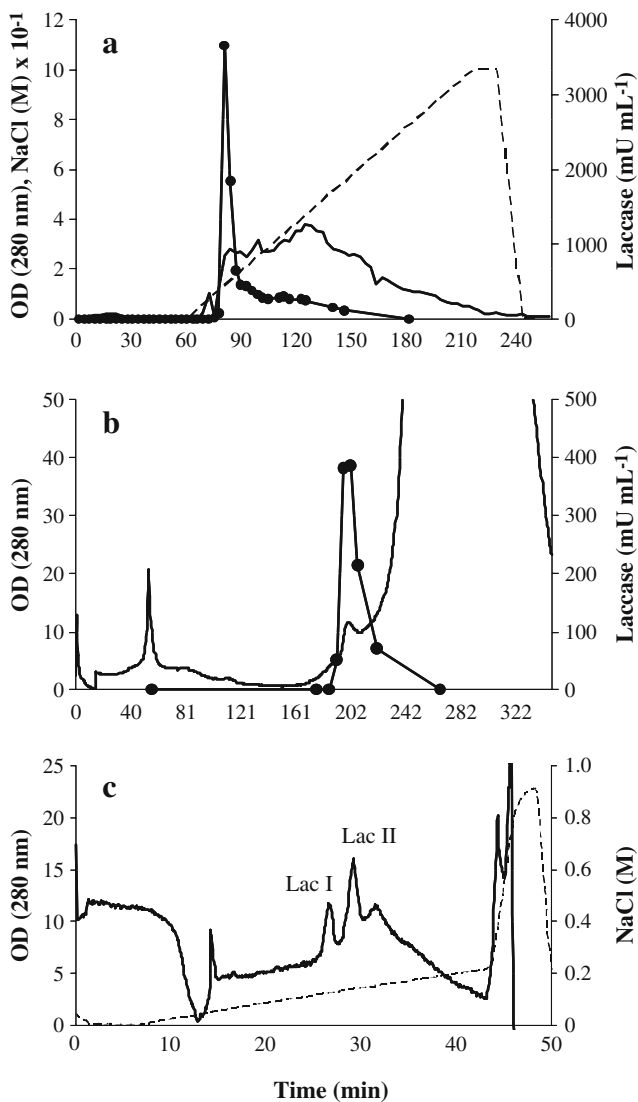


Fig. 3 Purification of laccases from *C. rigida* secreted in medium with WSFA and glucose: HiTrap-Q (a), Superdex 200 (b), and Mono-Q (c) chromatography. Absorbance at 280 nm (solid line), NaCl gradient (dashed line), and laccase activity (filled circle) are indicated

WSFA transformation by laccase

The incubation of WSFA for 24 h with a laccase preparation (LacI+LacII), revealed a 42% free phenol reduction (from 368.9 to 214.4 $\mu\text{g mL}^{-1}$), estimated by Folin method. The analysis of these samples in a size-exclusion column showed that the main peak, corresponding to low molecular mass, decreased, while the peak corresponding to higher molecular mass increased (Fig. 5).

Identification of *C. rigida* laccase gene expressed in presence of WSFA

In order to obtain partial genomic sequences encoding the laccase isoenzymes involved in transformation of WSFA, PCR assays were carried out following the protocols described above using cDNA as template, and a single 1.2 kb band was obtained. More than 10 clones containing the 1.2 kb insert were sequenced, resulting all of them in

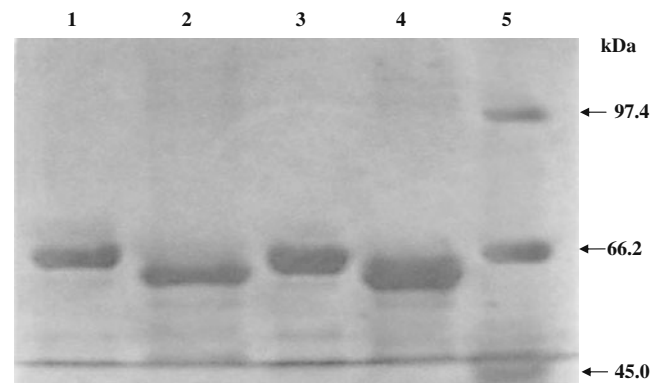


Fig. 4 Electrophoresis gel of purified *C. rigida* laccases secreted in medium with WSFA and glucose: LacI (lane 1), deglycosylated LacI (lane 2), LacII (lane 3), deglycosylated LacII (lane 4), and molecular mass standard (lane 5)

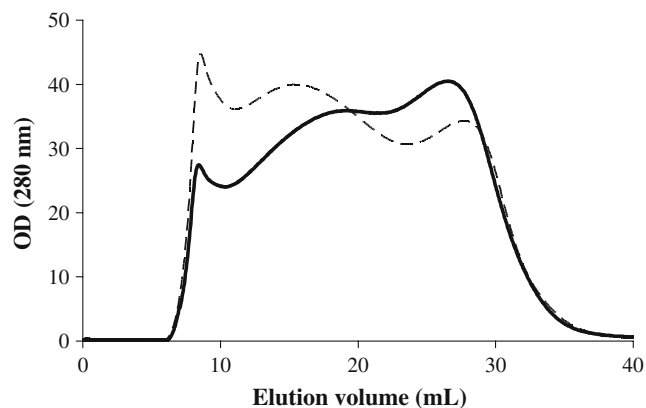


Fig. 5 Molecular mass distribution of phenols from WSFA estimated as profiles of absorbance at 280 nm by size-exclusion chromatography, before (*solid line*) and after treatment for 24 h with a *C. rigida* laccase preparation (*dashed line*)

the same sequence. Thus, a single sequence was obtained, which showed the highest homology (87%) with the *lcc1* laccase gene from *Trametes* sp. C 30 (GenBank accession number no. AF491759.1) and *lac1* and *lacA* laccase genes from *C. gallica* (86% and 85% of homology) (GenBank accession number no. DQ431716.1 and AY875867.1, respectively). The sequence obtained, which included the N-terminus coding region, was used to design the primer CR3, corresponding to the exact sequence of the N-terminus, and the sequences showing highest homologies were then used to design the primer CR2 to obtain the flanking region corresponding to the C-terminus. A single band was obtained in PCR amplifications performed with either genomic DNA (~2.1 kb) or cDNA (~1.4 kb) and primers CR2 and CR3. Alignment of the genomic fragment and the corresponding cDNA laccase gene of *C. rigida*, named *lcc1*, showed a complete nucleotide match in their overlapping regions and indicated the presence of 11 exons. The gene was deposited in GenBank (accession number GQ377839).

The corresponding amino-acidic sequence of the cDNA showed the highest identity with *lac1* from *T. trogii* (90% identity and 94% similarity) (accession number CAC13040) and an identity of 89% with *C. gallica* laccase (94% similarity) (accession number ABD93940). The deduced protein presents 496 amino acids with a molecular mass of 66 kDa. The four copper domains, which are highly conserved in laccases (Hong et al. 2007), as well as the pattern of His-X-His repeats involved in the coordination of the trinuclear type-2/type-3 copper site, were found. The expected residues that coordinate the type-1 copper site were also observed: His 394, His 455, and Cys 450, involved in the trigonal coordination of type copper and Phe 460. Four potential N-glycosylation sites were also identified at positions 51, 54, 207, and 433, following the pattern of Asn-X-Ser/Thr.

Protein identification

The results of the digested LacI and LacII bands, analyzed by MALDI-TOF/TOF, showed no differences between tryptic fingerprints of both LacI and LacII isoenzymes. In addition, the sequencing of seven different peptides from each isoenzyme, which matched with the sequence deduced from *lcc1* cDNA, did not reveal any amino-acidic differences (data not shown).

Discussion

White-rot fungi have developed a non-specific oxidative system, including different oxidoreductases such as laccases and peroxidases, low molecular mass metabolites, and activated oxygen species, to degrade lignin and other aromatic pollutants causing environmental problems (Schoemaker et al. 1991). During transformation of WSFA, laccase was the sole ligninolytic enzyme activity detected. This enzyme is present in most of basidiomycetes, having a significant role especially when they are secreted as the sole ligninolytic enzyme (Eggert et al. 1997; Saparrat et al. 2002; Jaouani et al. 2005). The activity levels of laccases produced by *C. rigida* in the cultures supplemented with WSFA were lower than those detected in a previous report, where the fungus was grown in basal medium with glucose and copper (Saparrat et al. 2002). This could be due either to the presence of high levels of free phenols in WSFA which might affect enzyme secretion or reduce its activity (Tomati et al. 1991; Tsioulpas et al. 2002) or to the absence of copper which could act as enzyme inductor.

Analytical isoelectric focusing of extracellular crude extracts from cultures of *C. rigida* growing in the presence of WSFA showed two laccase bands (Fig. 2). The isoenzymes secreted in WSFA supplemented with glucose were similar, although the presence of glucose in the media delayed the secretion of laccase activity. This delay could be due to glucose repression, since CreA consensus sequences have been found in the non-coding regions of laccases from other basidiomycetes (Mansur et al. 1998; Galhaup et al. 2002). All the laccase isoenzymes detected showed similar acidic pI to those reported in other white-rot fungi, including *Grammothele subargentea* (pI 3.5), *Cerrena unicolor* (pI 3.6 and 3.7 for LacI and LacII, respectively), and *Panus tigrinus* (pI 3.15) (Michniewicz et al. 2006; Quarantino et al. 2007).

It is well known that culture media composition may affect the secretion of laccases (Leontievsky et al. 1997). For example, different laccase isoenzymes were induced in *Pycnoporus sanguineus* growing in the presence of high concentrations of starch and other sugars (Dantán-González et al. 2008). LacII from *Pleurotus eryngii* was induced by

lignin and derived compounds added to basal medium with glucose (Muñoz et al. 1997). *Botryosphaeria rhodina* laccases were induced by soybean oil, Tween 80, or copper (Dekker et al. 2007). *Trametes versicolor* and *P. tigrinus* laccases were induced by xyloidine (Minussi et al. 2007; Quarantino et al. 2008). Although in the case of *C. rigida*, a previous report had shown that two laccase isoenzymes were produced in the basal medium with glucose and copper (Saparrat et al. 2002), one of the main objectives of this study was to identify the enzymes induced under transformation of WSFA. Most of laccases are monomeric glycoproteins with a molecular mass between 50 and 80 kDa (Thurston 1994; Baldrian 2006). Two *C. rigida* laccases purified from the cultures with WSFA are monomeric proteins, as deduced by their similar molecular mass determined by SDS-PAGE and size-exclusion chromatography. A slight difference in the pI values permitted their separation in Mono-Q column. Although the same isoenzymes had been also obtained from basal medium with glucose and copper (Saparrat et al. 2002), the yield of purification process in the media with WSFA was lower (2.2% and 2.6% final yield for LacI and LacII, respectively), possibly due to the low-activity level obtained in this medium. The laccase isoenzymes from WSFA cultures showed similar affinity for both DMP and ABTS, which were used as phenolic and non-phenolic model compound respectively, when compared to the respective values of laccases obtained from the basal medium with glucose and copper. Both DMP and ABTS are typical laccase substrates, but DMP was especially chosen since it is the most abundant volatile phenol found after thermal treatment of olive stones and solvent-extracted olive pulp (Petrov et al. 2008). Similar K_m values were also described for laccases from *Pycnoporus coccineus*, 27 and 36 μM for DMP and ABTS, respectively (Jaouani et al. 2005), and *Daedalea quercina*, 48 and 38 μM for DMP and ABTS, respectively (Baldrian 2004). Zymograms carried out by isoelectrofocusing with the extracellular crude extracts from WSFA cultures suggested that the isoenzymes secreted in these conditions were the same to those secreted in the basal medium with glucose and copper. This was also supported by the kinetic constant values as well as the data regarding molecular mass described above.

Since toxicity of the “alpeorujo” is mainly due to hydrosoluble compounds which include free phenols as well as glycosylated phenols, secoiridoids, and flavonoids (Capasso et al. 1995), we analyzed the effect upon WSFA of the extracellular laccases secreted by *C. rigida* on WSFA. Aranda et al. (2006) previously reported the reduction in the levels of the main phenolic compounds from “alpeorujo” such as tyrosol and hydroxytyrosol by *C. rigida*, suggesting the possible participation of its laccase activity in the process. The results obtained in this work

indicate that laccases reduced levels of soluble aromatics including free phenols via polymerization of these compounds, as deduced from gel permeation profiles of treated WSFA, which showed a reduction of the peak area corresponding to low molecular mass and an increase of the higher molecular mass peak (Fig. 5). Similar results regarding molecular mass profiles have been reported by using free or immobilized crude laccase from *P. coccineus* or *L. edodes* using other effluent from the olive oil production (D'Annibale et al. 2000; Jaouani et al. 2005; Berrio et al. 2007). Since reduction of phytotoxicity and microtoxicity of “alpeorujo” is directly related to polymerization of phenols present in this residue (Aranda et al. 2007; Saparrat et al. 2010), we can conclude that the isozymes purified in this work are directly involved in the process of transformation of WSFA, and therefore they might be, at least at some extent, related to its detoxification.

Although two laccase isoenzymes have been isolated from *C. rigida* grown in presence of WSFA, a single laccase cDNA could be identified and cloned. The *C. rigida* laccase amino-acidic sequence deduced from this cDNA sequence showed highest homology with *T. trogii* laccase, and the deduced amino-acidic sequence showed all the domains observed for blue laccase from other basidiomycetes (Colao et al. 2003). The presence of two different laccase bands cannot be due to variants of the same enzyme with distinct glycosylation, since after deglycosylation of LacI and LacII, two bands were still visible in the gel (data not shown). Thus, the most plausible hypothesis is that each band would correspond to different allelic variants, since we are studying a dikaryotic fungal strain, although only one could be recovered by PCR. This would be supported by the physico-chemical properties of both LacI and LacII, the same amino-acidic N-terminus determined in both isoenzymes in the basal medium with glucose and copper (Saparrat et al. 2002) and the same peptide mass fingerprint obtained for both isoenzymes by MALDI-TOF MS. Although no differences were found in the sequenced peptides analyzed in both isoenzymes, small changes in their electrophoretic mobility could have been explained if the two protein bands corresponded to allelic variants, according to the results reported in other fungal species where a substitution in only three amino acids changed the isoelectric point (Ruiz-Dueñas et al. 1999).

In summary, we have identified two laccase isoenzymes from *C. rigida* directly involved in the transformation of “alpeorujo” via polymerization of phenols. The isoenzymes are identical to those produced by *C. rigida* in the basal medium with glucose and copper, and are encoded by the *lcc1* gene, which is expressed during “alpeorujo” transformation. Since further research is needed to completely understand the mechanisms of detoxification, as well as to optimize the process to be integrated at the industrial level,

we have recently initiated attempts to explore the transcriptional response to different environmental conditions of *lcc1* gene.

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