Archives of Biochemistry and Biophysics 474 (2008) 213–219

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Comparative characterization of four laccases from *Trametes versicolor* concerning phenolic C–C coupling and oxidation of PAHs

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ARTICLE INFO

Article history:
Received 19 February 2008
and in revised form 7 March 2008
Available online 14 March 2008

Keywords:
Laccase
Trametes versicolor
Polycyclic aromatic hydrocarbons
Oxidation
C–C coupling
Site-directed mutagenesis

ABSTRACT

The laccase genes *lccα*, *lccβ*, *lccγ* and *lccδ* encoding four isoenzymes from *Trametes versicolor* have been cloned and expressed in *Pichia pastoris*. Biochemical characterization allowed classification of these laccases into two distinct groups: Lccα and Lccβ possessed higher thermal stability, but lower catalytic activity towards 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) compared to Lccγ and Lccδ. Activities of the laccases were quite different as well. Laccase Lccδ showed highest phenolic C–C coupling activity with sinapic acid, but lowest oxidizing activity towards polycyclic aromatic hydrocarbons (PAHs). Highest activity towards PAHs was observed with Lccβ. After 72 h, more than 80% of fluorene, anthracene, acenaphthene and acenaphthylene were oxidized by Lccβ in the presence of ABTS. Investigation of the structural basis of the different activities of the laccases demonstrated the impact of positions 164 and 265 in the substrate binding site on oxidation of PAHs.

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2.) are very attractive enzymes for biocatalysis as they work with molecular oxygen at ambient temperatures, produce water as a by-product and do not require the costly co-factors NADH or NADPH as many other oxidoreductases. Based on their structural and mechanistic properties, laccases belong to the multicopper oxidase family. They are widely distributed among fungi, higher plants [1] and also bacteria [2]. In fungi laccases are involved in degradation of lignin [3], pigment production [4] and pathogenesis [5]. In plants they participate in lignification [6]. Laccases oxidize electron-rich compounds such as phenols, anilines, thiols, *N*-hydroxyl groups and many others by radical mechanism through the concomitant reduction of molecular oxygen to water [7]. Products of the laccase-catalyzed reactions, phenolic radicals, can undergo self-assembling by C–C and C–O coupling and build dimers or oligomers [8–10].

Laccases from the white rot fungus *Trametes versicolor* belong to the well studied laccases. A phylogenetic analysis of the laccases from *T. versicolor*, whose sequences have been reported so far, revealed four isoenzyme groups α, β, γ and δ [11]. Laccase genes within each group shared at least 97% sequence identity and might represent just allelic variants. Several publications have described the utilization of mixtures of different *T. versicolor* laccases for degradation of polycyclic aromatic hydrocarbons (PAHs), polychlorophenols and other phenolic compounds [12–15]. Besides that, C–C coupling of sinapic acid using a crude enzyme preparation of *T. versicolor* has also been reported [10,16,17]. However, a comparison of biochemical properties and enzymatic activities of purified laccases' isoenzymes from *T. versicolor* is still missing.

In the present work, we describe cloning, expression and comparative characterization of laccases from all four isoenzyme groups of *T. versicolor* concerning their biochemical properties, phenolic C–C coupling activity and oxidation efficiency towards PAHs.[1] Further we investigated the influence of the amino acid residues at positions 164 and 265 in the substrate binding sites on the activity of different laccases.

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[1] *Abbrevations used:* ABTS; 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); PAHs, polycyclic aromatic hydrocarbons; BMM, buffered minimal medium; BMGY, buffered glycerol-complex medium; MTBE, methyl *tert*-butyl ether.

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doi:10.1016/j.abb.2008.03.009

Material and methods

cDNA synthesis

RNA was isolated from a culture of *T. versicolor* 3086 (DSMZ, german collection of microorganisms). The fungus was grown in 20 ml synthetic medium [18] with 6.6 mg/l $CuSO_4$ at 28 °C and 150 rpm. After 7 days of growth the mycelium was harvested and RNA was isolated using the RNeasy Plant Mini Kit according to the manufacturer's instructions.

PCR primers for cDNA amplification of *lccα* were designed based on the sequence of *lcc1* (GenBank Accession No. AY693776) [11], for *lccβ* based on *lcc2* (GenBank Accession No. Y18012) [19], for *lccγ* based on *CVLG1* (GenBank Accession No. D84235) [20] and for *lccδ* based on *lcc1* (GenBank Accession No. X84683) [21]. The primer sequences are listed in Table 1. The first strand cDNA was synthesized from total RNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo-dT primers and Lccδ reverse primer, respectively. Laccase genes were amplified from single-stranded cDNA using Lccα, Lccβ, Lccγ and Lccδ forward and reverse primers and the following PCR program: 94 °C (4 min, once), 94 °C (1 min), 60 °C (1 min), 72 °C (4 min), 40 cycles and 72 °C (4 min, once). PCR products of *lccα*, *lccβ* and *lccδ* were digested with HindIII and EcoRI, the PCR product of *lccγ* with HindIII and XhoI, respectively, cloned into pYES2 vector and sequenced.

The native signal peptides of the laccase genes were replaced by the α-mating factor signal peptide of *Saccharomyces cerevisiae* by overlap extension PCR. The α-mating factor signal peptide was amplified from the pPICZαA vector with the α-factor forward and reverse primer. The laccase genes were amplified without the native signal peptide, but with an overlap to the 3′ end of the α-mating factor signal peptide at the 5′ end of the forward primers. The reverse primers were the same as described above with exception of *lccγ* reverse primer Lccγ_XbaI. The laccase genes and the α-mating factor signal peptide were fused by overlap extension PCR using the α-factor forward primer and the reverse primers described above (for *lccγ* Lccγ_XbaI). The PCR products were digested with HindIII and EcoRI (*lccα*, *lccβ*, *lccδ*) or with HindIII and XbaI (*lccγ*), cloned into pYES2 and sequenced.

Cloning into P. pastoris expression vector

The laccase genes were amplified by PCR from the pYES2 plasmids and cloned into pPICZA vector. For amplification of laccase genes with their native signal peptide Lccα_pPICZA, Lccβ_pPICZA, Lccγ_pPICZA and Lccδ_pPICZA forward primers were used. For amplification of laccase genes with the α-mating factor signal peptide the α-factor_pPICZA forward primer was applied. Reverse primers were the same as described above (for *lccγ* Lccγ_ XbaI). PCR products were digested with *Bsp*119I and EcoRI (*lccα*, *lccβ*, *lccδ*) or with *Bsp*119I and XbaI (*lccγ*), ligated with the digested pPICZA vector and sequenced. *P. pastoris* X-33 cells were transformed with PmeI or SacI linearized plasmids or empty pPICZA vector according to the *Pichia* expression vectors manual (Invitrogen). Transformants were picked from YPDS plates supplemented with zeocin (100 μg/ml) and screened for production of laccase by transferring the colonies on minimal methanol plates containing $CuSO_4$ (0.3 mM) and ABTS (0.2 mM). Transformants with the highest laccase production were identified by the largest green halo around *Pichia* colonies and used for further studies.

Table 1
Forward and reverse primer sequences used for amplification and site-directed mutagenesis of laccase genes

Name	Direction	Sequence
Lccα	Forward	CCATACAAGCTTATGGGTCTGCAGCGGTTCAGCTTC
	Reverse	GGAGTGAATTCTCACTGGTCGGCCTCGCTC
Lccβ	Forward	CCATACAAGCTTATGTCGAGGTTTCACTCTCTTCTCGC
	Reverse	GGATAGAATTCTTACTGGTCGCTCGGGTCGAG
Lccγ	Forward	CCATACAAGCTTATGGGCAAGTTTCACTCCTTCGTGAAC
	Reverse	GGATAGAATTCTCAGAGGTCGGACGAGTCCAAAG
Lccγ_ XbaI	Reverse	CCTGTGTCTAGATCAGAGGTCGGACGAGTCCAAAG
Lccδ	Forward	CCATACAAGCTTATGGGCAGGTTCTCATCTCTCTGC
	Reverse	GGATAGAATTCTTAGAGGTCGGATGAGTCAAGAGCG
α-factor	Forward	CCATACAAGCTTATGAGATTTCCTTCAATTTTTACTGCTG
	Reverse	CAGCTTCAGCCTCTCTTTTCTC
Lccα_pPICZA	Forward	CTAATTATTCGAAACGATGGGTCTGCAGCGGTTCAG CTTC
Lccβ_pPICZA	Forward	CTAATTATTCGAAACGATGTCGAGGTTTCACTCTCTT CTCGC
Lccγ_pPICZA	Forward	CTAATTATTCGAAACGATGGGCAAGTTTCACTCCTT CGTGAAC
Lccδ_pPICZA	Forward	CTAATTATTCGAAACGATGGGCAGGTTCTCATCT CTCTGC
α-factor_pPICZA	Forward	CTAATTATTCGAAACGATGAGATTTCCTTCAATTT TTACTGCTG
Lccβ_L164V	Forward	CCGCATTCCCTGTGGGCGCCGACGCCAC
	Reverse	GTGGCGTCGGCGCCCACAGGGAATGCGG
Lccβ_F265A	Forward	GCCAACCCGAGCGCGGGTAACGTCGGG
	Reverse	CCCGACGTTACCCGCGCTCGGGTTGGC

Site-directed mutagenesis

Construction of L164V and F265A mutants of Lccβ was performed using the Stratagene™ Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. Mutations were introduced using Lccβ_L164V and Lccβ_F265A forward and reverse primers.

Expression and fermentation of laccases

Pichia pastoris transformants were grown overnight in 10 ml buffered glycerol-complex medium (BMGY, 10 g yeast extract, 20 g peptone, 0.1 M sodium phosphate buffer pH 6.0, 100 ml of 10% (v/v) sterile glycerol and 0.04 mg/ml biotin) at 30 °C and 180 rpm. Overnight cultures were harvested by centrifugation (1500g, 10 min). The pellets were resuspended in 50 ml buffered minimal medium (BMM, 13.4 g YNB, 0.1 M sodium phosphate buffer pH 6.0, 0.5% (v/v) methanol, 0.04 mg/ml biotin and 0.1–0.6 mM $CuSO_4$) to an OD_{600} of 1.0. *P. pastoris* transformants were cultivated at 17–30 °C and 180 rpm. Methanol (100%) was added daily (final concentration 0.5% (v/v)) to maintain induction. Samples were taken daily for spectrophotometric determination of cell growth and laccase activity.

The bioreactor cultivations were seeded with the content of shake flask cultures, growing over night at 30 °C and 180 rpm, up to an OD_{600} of 0.5. Cultivations were performed in a 7.5 l reactor (Infors, Botttmingen, Switzerland) containing basal salt medium (5 l, 45.5 g K_2SO_4, 37.5 g $MgSO_4 \cdot 7H_2O$, 31 g KOH, 2.35 g $CaSO_4{\cdot}2H_2O$, 66.75 ml H_3PO_4 (85%), 250 g glycerol, 0.5 ml antifoam solution 286, 4.35 mg biotin and 1.5 ml PTM_1 trace salts (one litre PTM_1 contains 6 g $CuSO_4{\cdot}5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4 \cdot H_2O$, 0.5 g CoCl, 20.0 g ZnCl, 0.02 g H_3BO_3, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 65.0 g $FeSO_4{\cdot}7H_2O$, 0.2 g biotin and 30 ml 6 N H_2SO_4)). The fermentation temperature was 30 °C, after induction it was shifted to 25 °C or 18 °C. The pH was maintained at 5.0 using NH_4OH (28%) and H_3PO_4 (10%). The airflow was kept at 10 l/min and the stirrer speed was adjusted between 800 and 1000 rpm. When the initial methanol concentration (0.5% (v/v)) in the culture broth was depleted, indicated by an abrupt increase in dissolved oxygen, methanol solution (20 g, with 1.2% (v/v) PTM_1) was automatically added.

Protein purification

Culture supernatants of the fermentation processes containing the laccases were obtained by centrifugation of the culture broths (10800g, 25 min). The supernatants were concentrated 29-fold by cross-flow ultrafiltration using a Millipore set-up according to the manufacturer's instructions with a silicone membrane (Pall, East Hills, NY, USA) of 10 kDa cut-off value. Laccases were purified using hydrophobic interaction chromatography. Phenylsepharose HP (Amersham Biosciences, Piscataway, NJ, USA) was equilibrated with 20 mM sodium acetate buffer (with 1.5 M $(NH_4)_2SO_4$, pH 5.0) (buffer A). A 10 ml sample (1:2.5 diluted and ammonium sulfate salted) was loaded onto the column at a linear flow rate of 5 ml/min. Afterwards, the column was washed with one column volume of buffer A. Laccases were eluted from the column with a step gradient using 20 mM sodium acetate buffer, pH 5.0. Fractions containing laccase activity were pooled. Next 0.5 ml of concentrated sample was applied on a Sephacryl S-300 HR gel filtration column (Amersham Biosciences) equilibrated with 20 mM sodium acetate buffer, pH 5.0. The chromatographies were performed at a flow rate of 0.25 ml/min. Fractions with laccase activity were pooled and concentrated using an Amicon ultrafiltration cell (Millipore, Billerica, MA, USA). Concentration of proteins was determined using Bradford assay [22]. Bovine serum albumin was used as a standard. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was done in a Minigel-Twin (Biometra, Goettingen, Germany). Proteins were visualised by silver staining.

Activity assays

Laccase activity was routinely assayed at room temperature using ABTS (0.5 mM) in 0.1 M sodium acetate buffer, pH 5.0. Activity was measured by monitoring the change in absorption at 420 nm with an extinction coefficient of 36 $mM^{-1}\,cm^{-1}$ [23]. One unit is defined as the amount of enzyme that oxidizes 1 μmol of substrate per minute. For activity measurements at various pH values, laccase activities were assayed in 0.1 M citrate phosphate buffer at pH values of 1.5–8.0. Stability of enzymes at pH 2.0, 3.0, 4.0 and 5.0 were determined by incubating laccase solutions (0.5 μM) in 0.1 M citrate phosphate buffer, pH 2.0–5.0 at room temperature for 24 h. After 1, 2, 3, 4, 5, 8, 15, 20 and 24 h samples were taken and ABTS oxidation activity was measured. For determination of ABTS oxidation activity at increased temperatures laccase activities were measured at various temperatures between 25 and 90 °C in 0.1 M sodium acetate buffer, pH 5.0 for 5 min. For thermostability measurements, laccase solutions (0.5 μM) were incubated at 25, 40, 60 and 80 °C in 0.1 M sodium acetate buffer, pH 5.0. After 20 min ABTS oxidation activity was determined at room temperature. Kinetic constants were determined in 0.1 M sodium acetate buffer, pH 5.0 at room temperature. ABTS concentration was varied in range of 0.005–5 mM.