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Laccases of *Pleurotus sapidus*: Characterization and Cloning

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Peanut shells, a major waste stream of food processing, served as a renewable substrate for inducing the production of laccases by basidiomycetes. Of 46 surface cultures examined, 29 showed laccase activity under the experimental conditions. The edible fungus *Pleurotus sapidus* was selected as the most active producer, immobilized on the shells, and cultivated in the fed-batch mode. A continuous rise in laccase activity was found, indicating the inducibility of laccase secretion by the peanut shells and the reusability of the mycelium. Two laccase isoenzymes were purified by decoupled 2-D electrophoresis, and amino acid sequence information was obtained by electrospray ionization tandem mass spectrometry. cDNAs of the corresponding gene and another laccase were cloned and sequenced using a PCR-based screening of a synthesized *P. sapidus* cDNA library. Data bank searches against public databases returned laccases of *P. ostreatus* and *P. sajor-caju* as the best hits. The potential use of laccases by the food industry is discussed.

KEYWORDS: Basidiomycete; *Pleurotus sapidus*; fungi; extracellular enzymes; laccase; cDNA library

INTRODUCTION

Pleurotus sapidus is a member of the oyster mushroom family. Because of their pleasant flavor and high nutritional value, oyster mushrooms are highly valued as edible fungi all around the world (*1*). Beyond their alimentary use, *Pleurotaceae* have been subject of larger research programs, as they secrete a broad set of extracellular enzymes when cultivated on lignified biopolymers (*2*, *3*). The best investigated group of these extracellular enzymes are laccases (E.C. 1.10.3.2), phenol oxidases that reduce oxygen to water and simultaneously perform a one-electron oxidation of various aromatic substrates (*4*). Laccases have found a number of applications, such as bleaching in the textile and dye industry, wood composite production, and bioremediation. Further applications in the paper and food industries and for the enzymatic conversion of chemical intermediates have been suggested (*5*−*9*). To satisfy the continuously increasing demand for fungal laccases, the enzymes are typically produced in submerged cultures, using waste streams of the agricultural industries as a carbon source. In previous investigations, for example, wheat straw (*10*), banana pseudostems (*11*), or coffee pulp (*12*) served as starting materials rich in lignocellulose. In solid-state cultures of *Trametes hirsuta*, grape seeds efficiently induced laccase production (*13*). In the present study, numerous basidiomycetes were screened for laccase secretion using peanut (*Arachis hypogaea* L.) shells as the substrate and enzyme inducer. A lignin content between 23% and 32% and a cellulose content of about 40% turn peanut shells into an appropriate substrate for the cultivation of basidiomycetes. A yearly production of peanuts of approximately 33 million tons worldwide ensures an almost unlimited availability (*14*).

MATERIALS AND METHODS

Chemicals. The constituents of nutrient media were purchased from Merck (Darmstadt, Germany), Fluka (Neu-Ulm, Germany), Riedel-de Haën (Seelze, Germany), and Sigma-Aldrich (Taufkirchen, Germany). Chemicals and materials for electrophoresis were obtained from Serva (Heidelberg, Germany) and Bio-Rad (München, Germany). Solvents were provided by BASF (Ludwigshafen, Germany) and Baker (Deventer, The Netherlands). All solvents were distilled before use. High-purity water was prepared with an E-pure water purification system (Barnstead, Dubuque, IA).

Fungi. The fungal strains (cf. **Table 1**) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), from the Centraalbureau voor Schimmelcultures (CBS), and from the American Type Culture Collection (ATCC).

Activity Screening on Agar Plates. Stock cultures of the fungal strains were maintained on standard nutrition agar plates [30.0 g L^{-1} D-(+)-glucose·H_2O; 4.5 g L^{-1} L-asparagine·H_2O; 1.5 g L^{-1} KH_2PO_4; 0.5 g L^{-1} $MgSO_4$; 3.0 g L^{-1} yeast extract; 15.0 g L^{-1} agar agar; 1.0 mL L^{-1} trace element solution containing $CuSO_4$·$5H_2O$ (0.005 g L^{-1}), $FeCl_3$·$6H_2O$ (0.08 g L^{-1}), $ZnSO_4$·$7H_2O$ (0.09 g L^{-1}), $MnSO_4$·H_2O (0.03 g L^{-1}), and EDTA (0.4 g L^{-1}); the pH was adjusted to 6.0 with 1 N NaOH prior to sterilization].

For the activity screening, the agar plates contained 10.0 g L^{-1} D-(+)-glucose·H_2O, 0.3 g L^{-1} l-asparagine·H_2O, 3.0 g L^{-1} malt extract, 15.0 g L^{-1} agar agar, 10 mL L^{-1} 1 M sodium phosphate buffer (pH 5.2), 100 mL L^{-1} saline solution **1** (20.0 g L^{-1} KH_2PO_4, 7.1 g L^{-1}

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Table 1. Laccase Activity[a] of Selected Basidiomycetes in Surface Cultures on Agar Plates (–) and on Agar Plates Supplemented with Peanut Shells (+)

	laccase activity on culture day							
	7		14		21		28	
species/strain[b]	+	–	+	–	+	–	+	–
Bjerkandera adusta CBS 414.48	0	0	1	0	1	0	1	0
Clitocybe lignatilis FSU C 31-2	0	0	0	0	2	1	2	0
Cyathus earlei CBS 440.80	3x	3x	3	3	1	0	0	0
Cyathus helenae CBS 372.80	1	2	3	3	2	3	1	2
Cyathus julietae CBS 373.80	1	1	2	2	0	0	0	0
Cyathus limbatus CBS 335.81	3x	3	3	3	2	2	3	3
Cyathus pallidus CBS 376.80	3	3	3	3	3x	3x	3x	1x
Cyathus striatus DSMZ 1652	2x	2x	3x	3x	3	2	3	1
Ganoderma applanatum CBS 250.61	1x	0	0	0	1	0	0	0
Grifola frondosa CBS 480.63	3	1	2x	3	3	2	3	1
Hericium erinaceus CBS 260.74	2	2	2	2	0	1x	1x	1
Hypomyces odoratus CBS 764.68	0	0	1	0	1	1	1	0
Ischnoderma benzoinum CBS 311.29	3	0	3x	1	2	3	2	2
Lentinula edodes FSU A 20-8	1x	1	3x	1	1	1	0	0
Lentinula edodes CBS 225.51	2	2	0	0	0	1	0	3
Lentinula edodes CBS 389.89	0	3	0	3	0	2	0	0
Lentinus lepideus CBS 450.79	2	0	3	0	2	0	3	0
Lepista irina CBS 458.79	2	1x	2x	3	3	2	3	3
Lepista nuda CBS 300.58	2x	3x	3x	3	3x	3x	3x	2
Marasmius alliaceus CAS 413	1	0	1	0	0	1x	2	0
Marasmius scorodonius CBS 850.87	2	2	2x	2x	3	2	2	2
Marasmius scorodonius CBS 166.44	3	3x	3x	2	3	3	3	3
Marasmius scorodonius CBS 137.83	3x	1	2	1x	3	3	3	3
Meripilus giganteus CBS 561.86	3x	2x	3	3	2	3	3	1
Pholiota squarrosa DSMZ 5127	3	2	2	0	2	2	1	0
Pleurotus sanguineus CBS 614.73	2	2	2	2	2	2	0	1
Pleurotus sapidus DSMZ 8266	3x	2x	3x	1	3	3	3[c]	3
Trametes suaveolens DSMZ 5237	2	0	1	0	2	1	1	1
Trametes versicolor DSMZ 11269	2	2x	2	2	3	3	1	3

[a] 0 = no observable activity, 1 = low, 2 = medium, and 3 = high activity. x indicates laccase activity in the surrounding of the mycelium. [b] A complete list of all 46 species screened is available upon request. [c] Color reaction localized adjacent to the peanut shells.

$MgSO_4 \cdot 7H_2O$, 1.1 g L^{-1} $CaCl_2 \cdot 2H_2O$), and 10 mL L^{-1} saline solution **2** [10.5 g L^{-1} NTA, 7.0 g L^{-1} NaCl, 3.5 g L^{-1} $MnSO_4 \cdot H_2O$, 0.7 g L^{-1} $FeSO_4 \cdot 7H_2O$, 0.7 g L^{-1} $CoSO_4 \cdot 7H_2O$, 0.7 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.07 g L^{-1} $CuSO_4 \cdot 5H_2O$, 0.07 g L^{-1} $KAl(SO_4)_2 \cdot 12H_2O$, 0.07 g L^{-1} H_3BO_3, 0.07 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$]. The pH was adjusted to 5.2 with 1 M HCl prior to sterilization. To induce enzyme secretion, 200 mg of ground and sterilized peanut shells (without seed coat, particle size 1.0−1.4 mm) were added to the plates before they solidified. The production of extracellular laccase activity was monitored by the formation of a purple color upon addition of a 1.6% (m/v) ethanolic solution of α-naphthol (*15*).

Laccase Production in Submerged Cultures of *P. sapidus*. For preparation of precultures, 14-mm-diameter agar plugs from the leading mycelial edge of the *P. sapidus* (DSMZ 8266) stock culture were transferred into 100 mL of standard nutrition solution and homogenized using an Ultra Turrax dispersing tool (IKA-Werke, Staufen, Germany). After cultivation for 7 days at 24 °C and 150 rpm, the cultures were centrifuged (3300*g*, 4 °C, 10 min) and homogenized, and 25 g of the homogenate were resuspended in 10 mL of sterile solution **A** [10 mL L^{-1} 1 M sodium phosphate buffer (pH 5.2), 100 mL L^{-1} saline solution **1**, 10 mL L^{-1} saline solution **2**, pH adjusted to 5.2 with 1 M HCl]. This suspension was used to inoculate 7 g of peanut shells in 1-L Erlenmeyer flasks. After 7 days of static incubation at 24 °C, 250 mL of nitrogen-deficient nutrient solution {5% (v/v) sterile filtered solution **B** [200 g L^{-1} D-(+)-glucose, 4 g L^{-1} ammonium tartrate, 40 mg L^{-1} thiamine hydrochloride], 95% (v/v) solution **A**} was added, and the cultures were grown at 24 °C and 100 rpm.

Protein Concentration. The protein concentration was estimated by the method of Bradford (*16*) using bovine serum albumin as a standard.

Electrophoresis. IEF (isoelectric focusing) polyacrylamide gel electrophoresis was performed on a Multiphor II system (Pharmacia LKB, Uppsala, Sweden) using Servalyt Precotes precast gels with an immobilized pH gradient from pH 3 to 6 (Serva, Heidelberg, Germany) for 3500 V h (2000 V, 6 mA, 12 W). The isoelectric points of the laccases were estimated using a low-pI calibration kit from 2.8 to 6.5 (Pharmacia LKB). Gels were Coomassie or activity stained using 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS). For the transfer of protein spots from IEF to SDS (sodium dodecyl sulfate) gels, the unstained enzymes were excised from the IEF gels and prepared for the SDS−PAGE (sodium dodecyl sulfate−polyacrylamide gel electrophoresis) experiments as described below.

SDS−PAGE analyses were performed using a 12% polyacrylamide separation gel (*17*). Gel pieces excised from IEF gels were prepared by adding 20 μL of loading buffer [0.1 M Tris/HCl (pH 6.8), 0.2 M DTT, 4% SDS, 20% glycerol, 0.2% bromophenol blue] and boiling for 10 min. For molecular mass determinations, marker proteins from 200 to 14.3 kDa (Roth, Karlsruhe, Germany) were used.

Native PAGE was performed under nondenaturing conditions. Samples were prepared by mixing 1:1 (v/v) with loading buffer [0.05 M Tris/HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue], and the gels were activity stained with ABTS.

Laccase Activity Assay. The activity assay was based on the laccase-catalyzed oxidation of ABTS to the greenish blue cation radical $ABTS^{\bullet+}$ (*18*). The time-dependent increase of absorbance was monitored at 420 nm and 27 °C using a spectral photometer equipped with a thermostated magnetic stirrer unit (Lambda 12, Perkin-Elmer, Überlingen, Germany). Using an extinction coefficient (ϵ_{420}) of 36 000 L mol^{-1} cm^{-1}, enzyme activity was calculated after an assay time of 2 min according to the following equation

$$A\ (\mathrm{U\ L^{-1}}) = \frac{\Delta E V_t}{0.036 V_s}$$

where U is the enzyme activity catalyzing the oxidation of 1 μmol of ABTS per minute, ΔE is the increase in absorbance per minute, V_t is the total volume in the cuvette (mL), and V_s is the sample volume in the cuvette (mL).

MALDI-TOF-MS Analysis of Tryptic Peptides. The laccase spots were excised from SDS−PA gels, dried, and digested with trypsin. The resulting peptides were extracted and purified according to standard protocols. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was employed to obtain peptide mass fingerprints. A sample (0.5−1 μL) of each peptide solution was mixed with the same volume of a saturated matrix solution of α-cyano-4-hydoxycinnamic acid in 0.5% HCOOH/65%MeOH, spotted onto the target, and dried at room temperature. The molecular masses of the tryptic peptides were determined in the positive-ion mode on a Bruker Ultraflex time-of-flight mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) using an acceleration voltage of 20 kV. Peptide mass fingerprints (PMFs) obtained from MALDI-TOF-MS analyses were used for cross-species protein identification in public protein primary sequence databases. Mascot (Matrix Science Ltd., London, U.K.; http://www.matrix science.com) was employed for analysis of the MALDI data using the public databases NCBInr.

ESI-Tandem MS. A QTof II mass spectrometer (Micromass, Manchester, U.K.) equipped with a nanospray ion source and gold-coated capillaries (Protana, Odense, Denmark) was used for electrospray ionization (ESI) MS of peptides. For collision-induced dissociation experiments, multiple charged parent ions were selectively transmitted from the quadrupole mass analyzer into the collision cell (collision energy 25−30 eV for optimal fragmentation). The resulting daughter ions were separated by an orthogonal time-of-flight mass analyzer. The acquired MS−MS spectra were enhanced (Max. Ent. 3, Micromass) and used for the ab initio sequencing of tryptic peptides.

cDNA Synthesis and PCR Screening. Cell disruption was achieved by grinding mycelium (about 300 mg) of the fourth culture day under liquid nitrogen. For isolation of total RNA, a silica-gel-based membrane (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany) was applied. The integrity of the RNA was checked by denaturing formaldehyde agarose gel electrophoresis and ethidium bromide staining. cDNA was constructed using the SMART cDNA library construction kit according to the manufacturer's instructions (BD Biosciences, Heidelberg, Germany). Superscript II (Gibco BRL, Karlsruhe, Germany) RNase H^- point mutant MMLV reverse transcriptase was used for first-strand synthesis, and HotStarTaq Polymerase (Qiagen) was employed for PCR amplification of *P. sapidus* laccase cDNAs. PCR primers were deduced from amino acid sequences of tryptic peptides with the assistance of the primer3 algorithm (*19*). Amplification experiments of the specific cDNA fragment were performed at a 57 °C annealing temperature in a Master Cycler gradient (Eppendorf, Hamburg, Germany) with the following primer combination: 5′>GCAGGTAACCCCAACCTG-GA<3′)/(5′>CATCGTAAGCCGGGCAAAGT<3′). For the amplification of the entire laccase cDNA, the primers (5′> AAGCCCCACT-TGAGACATTCTC<3′)/(5′> GAACATGCAACGGCTTTCATTA<3′) were used at an annealing temperature of 57 °C. Additionally, the degenerate primers [5′>CA(CT)TGGCA(CT)GG(ACT)(CT)TCT-TC<3′] and {5′>GC(GT)CG(AG)A(CT)CCAGTAGTTG<3′] were used to screen the *P. sapidus* library for further laccase-encoding cDNAs. The entire cDNA of a second laccase was amplified with the primer combination (5′>CACCTACAACGATGTTTCCAG<3′) and (5′>GAACGAAAAGCTGTCGACTTG<3′). PCR products were extracted from agarose gels by use of a MinElute gel extraction kit (Qiagen), ligated into the pCR2.1-TOPO vector (TOPO TA cloning kit, Invitrogen, Carlsbad, CA), cloned into *E. coli* TOPO 10F′, and sequenced by the chain termination method (*20*) using a BigDye terminator cycle sequencing kit on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA).

RESULTS AND DISCUSSION

Forty-six basidiomycetous fungi were screened for laccase production on standard agar plates and, to induce enzyme secretion, on plates supplemented with ground peanut shells, a substrate representative of many lignified byproducts of food production from plants. Of 46 strains tested, 29 showed laccase activity under the experimental conditions (**Table 1**). With most of the positive species, laccase activity was detectable at the fungal mycelium and, additionally, in the area surrounding the mycelia. Only with *P. sapidus* was an intensive color reaction observed directly on the ground peanut shells. Therefore, this fungus was selected for the detailed investigation of the formation and inducibility of extracellular laccase activity.

For the production and biochemical characterization of laccases, *P. sapidus* was grown in submerged cultures. Sterile peanut shells served as a substrate and also as a carrier. In a fed-batch procedure, the culture supernatant was removed from the immobilized mycelium and replaced by fresh medium periodically. After each change of the medium, a significant rise in laccase activity from 125 U L^{-1} in the first cycles to more than 500 U L^{-1} in the fifth cycle was observed (**Figure 1**). A general induction of lignolytic enzymes might contribute to this effect as well as an increasing accessibility of the substrate's lignocellulose polymer with proceeding lignin

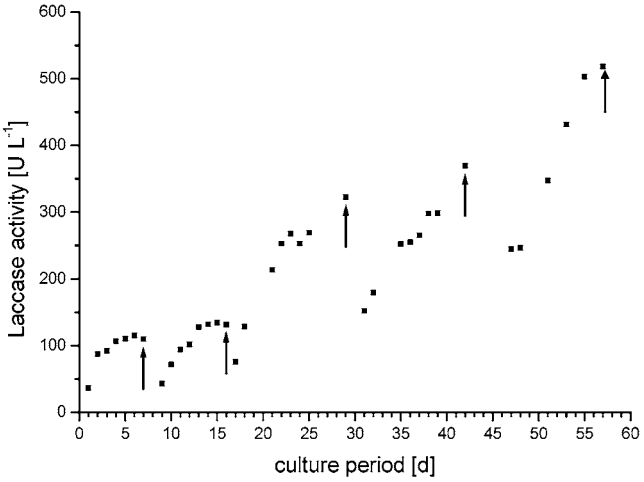

Figure 1. Laccase activity in culture supernatants of *P. sapidus* grown on peanut shells; the arrows indicate the exchange of the culture medium.

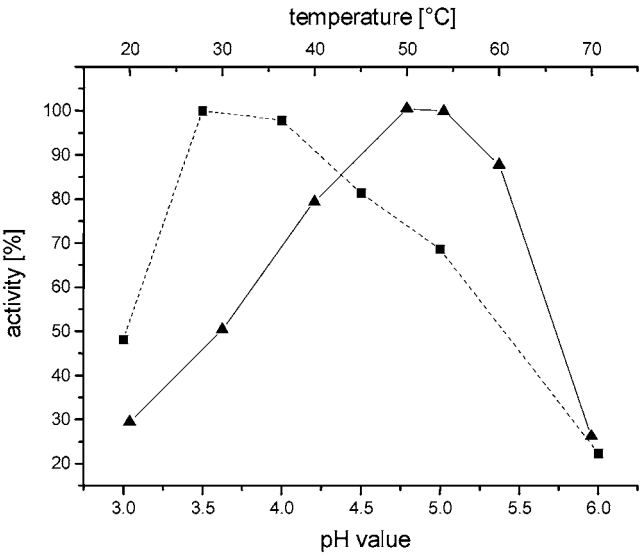

Figure 2. Temperature optimum (▲) and pH dependence (■) of *P. sapidus* laccases.

degradation. In reference cultures immobilized on glass wool instead of peanut shells, maximum laccase activities of 20−100 U L^{-1} were observed. For *P. eryngii*, Munoz et al. reported the induction of a laccase isoenzyme by wheat straw alkalilignin and several phenolic compounds (*21*). When laccases and versatile peroxidases of various *Pleurotus* species were employed for the degradation of phenolic and nonphenolic aromatic pollutants, wheat straw served as a growth substrate in liquid cultures (*8*). Reddy and co-workers used banana leaf biomass and banana pseudostems for the production of lignolytic enzymes by *P. ostreatus* and *P. sajor-caju* (*22*). In a screening of supports and inducers for laccase production by *Trametes versicolor*, barley bran led to the highest activity levels (*23*).

Using ABTS as a substrate, optimum laccase activity in the culture supernatant was determined at pH 3.5 and 50 °C (**Figure 2**). For the laccases POXA3a and POXA3b, derived from the closely related *P. ostreatus*, an optimal temperature of 35 °C (pH optimum 3.6) has been reported (*24*). To determine the isoelectric points of *P. sapidus* laccases, the samples were applied in several lanes alongside marker proteins on an IEF gel. After focusing, the gel was cut, and one part was subjected to Coomassie blue staining, and the second to activity staining with ABTS. The remaining third was stored for protein excision. Two major ABTS-active enzymes were detected at pI 4.0 and

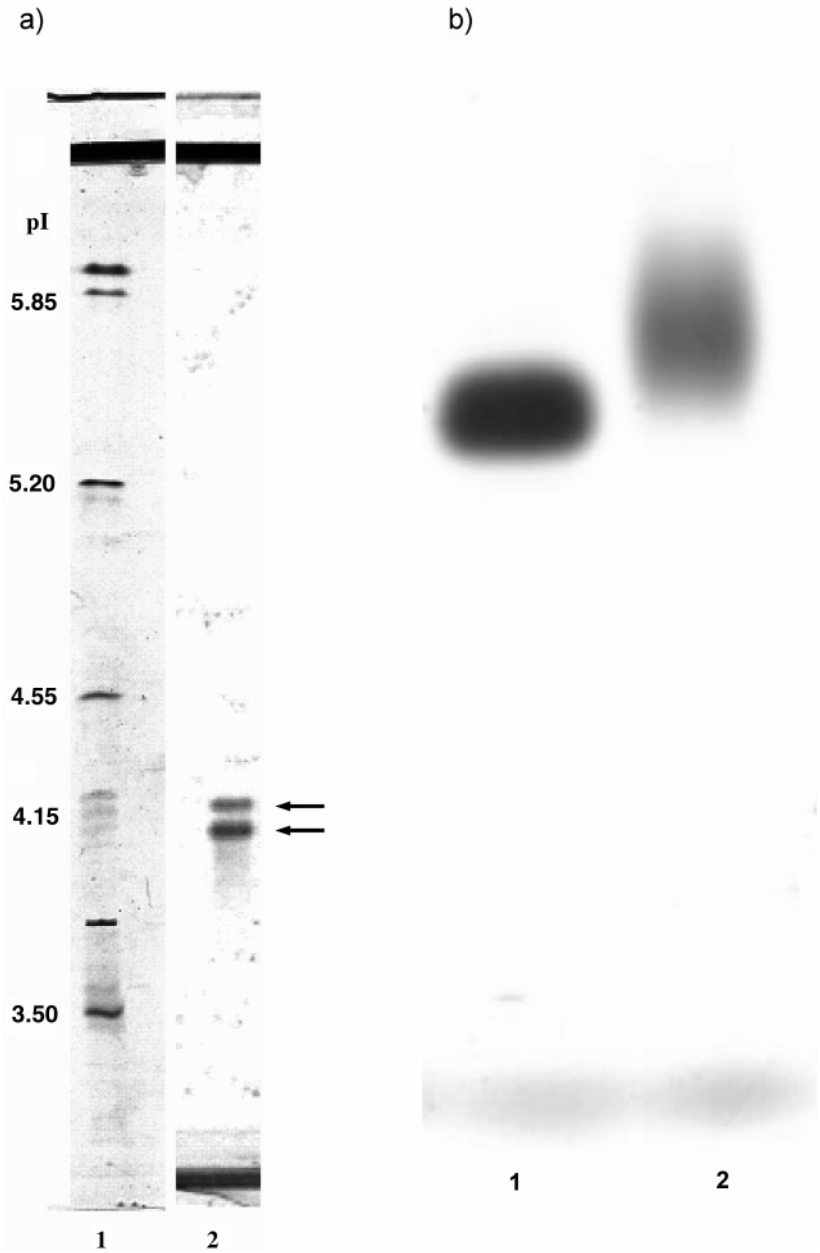

Figure 3. (a) Lane 1, pI marker proteins; lane 2, Zymogram showing laccase activity after IEF gel electrophoresis of culture supernatant of *P. sapidus*. (b) Native PAGE with laccase activity staining using ABTS as the substrate: lane 1, culture supernatant of *P. sapidus*; lane 2, reference (laccase C derived from *T. versicolor*).

4.1 (**Figure 3a**). The proteins appearing on the level of the ABTS-stained bands were excised from the unstained gel part and transferred to SDS−PAGE. On the SDS gel, a molecular weight of 66.8 kDa was assigned to the major protein band, and a second less-intense spot was detected at a molecular weight of 64.3 kDa. After in-gel tryptic digestion and peptide extraction, both proteins were shown to be homologous to laccase 3 (POXA3) of *P. ostreatus* and laccase 3 (LAC3) of *P. sajor-caju* by MALDI-TOF peptide mapping. Most probably, the two proteins represent isoforms. The secretion of laccases in multiple isoforms, depending on the environmental growth conditions, has been reported for several basidiomycetes (*25*). When the culture supernatant of *P. sapidus* was subjected to native PAGE, only one band was observed after activity staining with ABTS (**Figure 3b**). A similar observation has been reported for the atypical laccase isoenzymes of *P. ostreatus* (*24*). By means of electrospray ionization tandem MS, two tryptic peptides (APLTGGNPTGNPNLDVSLLR and TLCPAYDGLAPEFQ) were sequenced ab initio from the major laccase band excised from the denaturing SDS−PA gel (**Figure 4**). To identify the genetic information of the new enzyme, total RNA was extracted from *P. sapidus* at the point of maximum specific activity in the culture medium, and a cDNA library was constructed. A polymerase chain reaction (PCR) based approach was used to amplify the laccase-encoding gene, and primer sets were designed according to the reverse translated internal peptides. A 666 bp PCR product was amplified from the cDNA, which showed high homologies to various fungal laccases. By means of primer walking, a 1734 bp cDNA was finally cloned and sequenced. The obtained sequence contains an open reading frame (ORF) of 1566 bp, corresponding to a protein of 521 amino acids. A molecular weight of 57.4 kDa was calculated for the protein from the translated DNA sequence. The difference from the molecular mass observed on the SDS gel can be explained by a high degree of glycosylation. The ORF contains four potential *N*-glycosylation sites, three of which are predicted

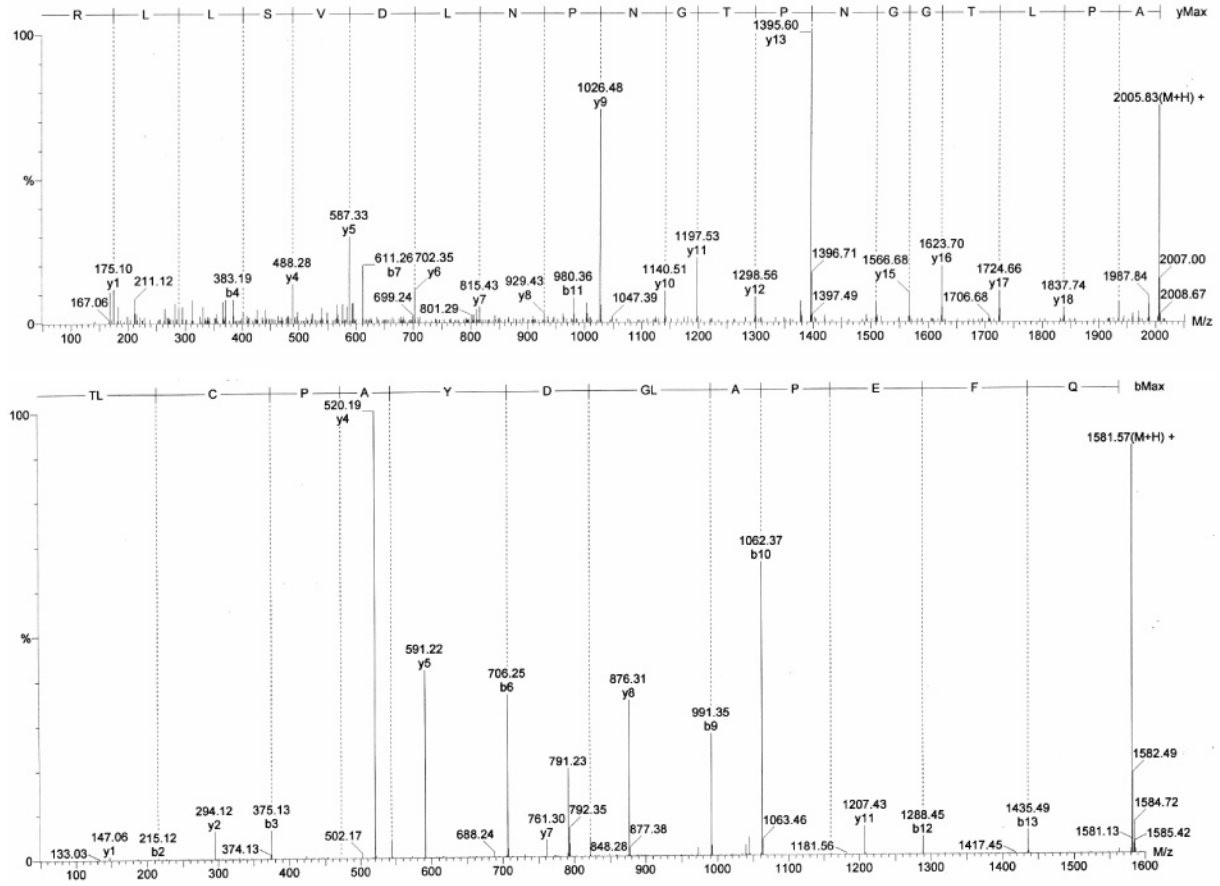

Figure 4. MS–MS spectra of two tryptic peptides ($m/z_{(MH^+)}$ = 2005.8 and 1581.6) derived from *P. sapidus* laccase. In the spectrum depicted in the upper panel, the carboxy terminal fragment ions of the y type have the highest intensity and have been assigned graphically, whereas in the peptide depicted in the lower panel, the amino terminal fragment ions of the b type are more complete and have therefore been assigned graphically. Because of the low-energy collision conditions used for the MS–MS experiments, leucine and isoleucine principally cannot be distinguished, i.e., L actually means L or I.

to be *N*-glycosylated (*26*). Mass spectrometry unequivocally confirmed the presence of a glycoprotein, as a series of molecular ions with mass increments of 162 Da typical of hexose residues was present in its tryptic peptide map. Furthermore, abundant carbohydrate-specific fragment ions were generated upon collision-induced dissociation during MS−MS experiments. In POXA1b derived from *P. ostreatus*, three of six putative *N*-glycosylation sites have been found to be posttranslationally modified (*25*). On the basis of lectin specificity, Palmieri et al. suggested the presence of high mannose and hybrid- or complex-type N-linked glycans for *P. ostreatus* laccases (*24*). Generally, a carbohydrate content varying between 22% and 45% is regarded to be characteristic for fungal laccases (*27*). The cDNA of the *P. sapidus* laccase displayed a high degree of homology with atypical laccase isoenzymes from two other *Pleurotus* species. Sequence similarity searches against public databases (*28*) returned laccase 3 (POXA3) of *P. ostreatus* (Q96TR4); *P. sajor-caju* lac3 (Q7Z8S4); and, with minor similarity, *A. bisporus* lac2 (Q12542) as the best hits. A comparison of laccases from *Pleurotus* species is presented in **Table 2**. On the cDNA level, the *P. sapidus* laccase showed a 90% homology to lac3 of *P. sajor-caju*, while the amino acid sequences of these two enzymes share a high sequence homology of 98%. The characteristic laccase signature sequences L1−L4, comprising one cysteine and 10 histidine residues involved in the binding of the four copper ions, are conserved in the *P. sapidus* laccase (**Figure 5**) (*29*). A 3-D model of the new laccase was calculated using the X-ray structure of *Trametes versicolor* laccase LacIIIb (pdb accession number 1KYA) (*30*) as a template (*31*) (**Figure 6**). The protein scaffold consists of three domains, which are characterized by antiparallel β-barrels. Thus, the overall structure is very close to that of the laccase Lac-Cc of *Coprinopsis cinerea* (*Coprinus cinereus*) (*32*).

Table 2. Laccases Produced by Different *Pleurotus* Species

species	accession no.	no. of amino acids	MW (Da) (calculated)	carbon source	ref
P. sapidus	Q6A1A1	531	56 739	peanut shells	this study
	Q4VY49	521	57 426	peanut shells	
P. ostreatus	O60199	533	57 980	potato dextrose, yeast extract	(*25*)
	Q96TR4	521	57 389	potato dextrose, yeast extract	(*24*)
	Q9UVY4	533	56 802	malt extract, yeast extract, glucose	(*33*)
P. sajor-caju	Q7Z8S2	522	57 054	not available	unpublished
	Q7Z8S3	532	56 473		
	Q7Z8S4	521	57 512		
	Q7Z8S5	532	57 506		
	Q7Z8S6	531	56 637		
P. eryngii	Q5MP11	531	56 606	not available	unpublished

To screen the *P. sapidus* cDNA library for further laccase-encoding sequences, degenerate PCR primers were derived from a sequence alignment of laccases derived from *Phlebia radiata* (Genbank accession number A20705), *Trametes versicolor* (TV44851), *Schizophyllum commune* (AB015758), *Pycnoporus cinnabarinus* (AF152170), and *Pleurotus ostreatus* (POAJ5018).

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Pslac           MVLSTKLAALVASLPFVLAATKKLDFHIRNDVVSPDGFERRAITVNGIFPGTPVILQKND 60
Popoxa3         MVLSTKLAALVASLPFVLAATKKLDFHIRNDVVSPDGFERRAITVNGIFPGTPVILQKND 60
Psclac3         MVLSTKLVALVASLPFVFAVTKKLDFHIRNDVVSPDGFERRAITVNGIFPGTPVILQKND 60
Ablcc2          MRFSNAFVLVAACISSVLADTKTFNFDLVNTRLAPDGFERDTVVINGEFPGTLVQVNKGD 60
                * :*. :. :.*.:. *:* **.::*.: *  ::****** ::.:** **** * ::*.*

Pslac           KVQINTINELTDPGMRRSTSIHWHGLFQHKTSGMDGPSFVNQ PIPPNSTFLYDFDTAGQ 120
Popoxa3         KVQINTINELTDPGMRRSTSIHWHGLFQHKTSGMDGPSFVNQCPIPPNSTFLYDFDTAGQ 120
Psclac3         KVQIKTINELTDPGMRRSTSIHWHGLFQHKTSGMDGPSFVNQCPIPPNTTFLYDFDTAGQ 120
Ablcc2          SVRIPVNNKLTSSTMRRSVSIHWHGFFQARTSGQDGPAFVNQCPQPPNTTFTYEFSVADE 120
                .*:* . *:**.. ****.******:** :*** ***:****** ***:** *:*..*.:

Pslac           TGNYWYHSHLSTQY DGLRGTFIVYDPNDPLKHLYDVDDESTIITLADWYHDLAPHAQNQ 180
Popoxa3         TGNYWYHSHLSTQYCDGLRGSFIVYDPNDPLKHLYDVDDESTIITLADWYHDLAPHAQNQ 180
Psclac3         TGNYWYHSHLSTQYCDGLRGSFIVYDPNDPLKHLYDVDDESTIITLADWYHDLAPHAQNQ 180
Ablcc2          SGTFWYHSHLSTQYCDGLRGAFVVYDPEDPLGHLYDVDDETTVITLAEWYHVLAPDINNE 180
                :*.:**************:*:****:*** ********:*:****:*** ***. :*:

Pslac           FFQTGSVPIPDTGLINGVGRFKGGPLVPYAVINVEQGKRYRFRLIQISCRPFFTFSIDNH 240
Popoxa3         FFQTGSVPIPDTGLINGVGRFKGGPLVPYAVINVEQGKRYRFRLIQISCRPFFTFSIDNH 240
Psclac3         FFQTGSVPIPDTGLINGVGRFKGGPLVPYAVINVEQGKRYRFRLIQISCRPFFTFSIDNH 240
Ablcc2          FFSSGIIPVQDSGLINGKGRFNGGPETPFAVVNVEQGKRYRFRVIAISCRPFFTFSVDNH 240
                **.:* :*: *:***** ***:*** .*:**:***********:* **********:***

Pslac           TFDAIEFDGIEHDPTPAQNIDIYAAQRASIIVNANQTIDNYWIRAPLTGGNPTGNPNLDV 300
Popoxa3         TFDAIEFDGIEHDPTPAQNIDIYAAQRASIIVHANQTIDNYWIRAPLTGGNPAGNPNLDI 300
Psclac3         TFDAIEFDGIEHDPTPAQNIDIYAAQRASIIVNANQTIDNYWIRAPLTGGNPAGNPNLDV 300
Ablcc2          NLTFMEADSVEHDPVEIQNVDIYAAQRVSVILNANQPVDNYWMRAPMTGGNPDRNPNLNI 300
                .:  :* *.:****.  **:*******.*:*::***.:****:***:*****  ****::

Pslac           SLIRAILRYKGAPAVEPTTVATTDGHKLNDADMHPIAQEGPGNLGTGPPDMAITLNIAQP 360
Popoxa3         SLIRAILRYKGAPAVEPTTVATTGGHKLNDAEMHPIAQEGPGNLGTGPPDMAITLNIAQP 360
Psclac3         SLIRAILRYKGAPAVEPTSVATTEGHKLNDADMHPIAQEGPGNLGTGPPDMAITLNIAQP 360
Ablcc2          SLTLAILRYKGAPEVEPTTVN-VPGHKLLDQEMHPIAQEGPGKLGDGPPDKHITLNIAQP 359
                **  ********* ****:*  . **** * :**********:** ****  ********

Pslac           NPPFFDINGISYLSPSVPVLLQMLSGARKPQDFLPSEQVIILPANKLIEVSIPGAGAHPF 420
Popoxa3         NPPFFDINGISYLSPSVPVLLQMLSGARKPQDFLPSEQVIILPANKLIEVSIPGAGAHPF 420
Psclac3         NPPFFDINGISYLSPSVPVLLQMLSGARKPQDFLPSEQVIILPANKLIEVSIPGAGAHPF 420
Ablcc2          NAPFFDINGISYISPTVPVLLQILSGAKRPEDVLPSEQIFFVPKNSLIEVNIPGEGAHPF 419
                *.**********:**:******:****::*:*.*****::::* *.****.*** *****

Pslac           HLHGHTFDIVRTSNSDVVNLVNPPRRDVLPINGGNTTFRFFSGNSGAWFLHCHIDWHLEA 480
Popoxa3         HLHGHTFDIVRTSNSDVVNLVNPPRRDVLPINGGNTTFRFFSGNSGAWFLHCHIDWHLEA 480
Psclac3         HLHGHTFDIVRVSNSDVVNLVNPPRRDVLPINGGNTTFRFFSGNSGAWFLHCHIDWHLEA 480
Ablcc2          HLHGHNFDVVLASNDDTFNFVNPPRRDVYPINGGNTTFRFFTDNPGAWFLHCHIDWHLEA 479
                *****.**:* .**.*..*:******** ************:.*.***************

Pslac           GLAVVFAERPAEVNDGEQAQIVTQDWRTLCPAYDGLAPEFQ 521
Popoxa3         GLAVVFAERPAEVNEGEQAQIVTQDWRTLCPAYDGLAPEFQ 521
Psclac3         GLAVVFAERPAEVNEGEQAQIVTQDWRTLCPAYDGLAPEFQ 521
Ablcc2          GLAIVFAEAPEDNVSGPQSQITPQDWLDLCPEYNAIEPEFQ 520
                ***:**** * :  .* *:**..***  *** *:.: ****
```

Figure 5. Translated amino acid sequence of *P. sapidus* laccase (the corresponding cDNA sequence was deposited at the EMBL database under the accession number AJ786026) and sequence alignment (*44*) with laccases of *P. ostreatus* (Popoxa3; accession number Q96TR4), *P. sajor-caju* (Psclac3; Q7Z8S4), and *Agaricus bisporus* (Ablcc2; Q12542). Peptides sequenced by ESI-tandem MS are printed in bold, potential *N*-glycosylation sites are depicted in italics, and the characteristic laccase signature sequence motifs L1–L4 are underlined.

As a result, a second laccase cDNA was cloned and sequenced. The obtained 1694 bp cDNA (deposited at EMBL Nucleotide Data Base under the accession number AJ786026) contains an open reading frame of 1596 bp, encoding a protein of 531 amino acids. Data bank searches returned *P. ostreatus* bilirubin oxidase (laccase, Q9UVY4) (*33*) with a sequence homology of about 94% on the amino acid level as the best hit. Although the overall sequence homology between the two laccases of *P. sapidus* was below 45%, all of the characteristic fungal laccase signature sequences were present in both deduced protein products. The protein product of the second laccase was not detected in the culture medium under the experimental conditions. However, the laccase gene family of *P. sapidus* can be clustered into at least two groups, as has been described recently for *C. cinerea* (*29*).

In recent years, an increasing number of potential applications of laccases have been reported from the field of food science. Laccases of *Botrytis cinerea* and *Trametes versicolor* served as biocatalysts for the oxidation of the sesquiterpene hydrocarbon valencene to nootkatone, the flavor-impact compound of grapefruit (*34*). As plant phenols are responsible for haze and sediment formation as well as for browning, astringency, and bitterness of fruit juices, laccase-mediated removal of phenolic compounds has become a promising technique for the juice industry (*35*, *36*). Additionally, laccase has been employed as an analytical tool to detect and quantify fruit- and vegetable-derived phenols. Amperometric biosensors using laccases as biorecognition elements have permitted the highly sensitive detection of phenols, e.g., in wastewater from an olive mill, and of polyphenolic compounds in red wine (*37*, *38*). Laccase

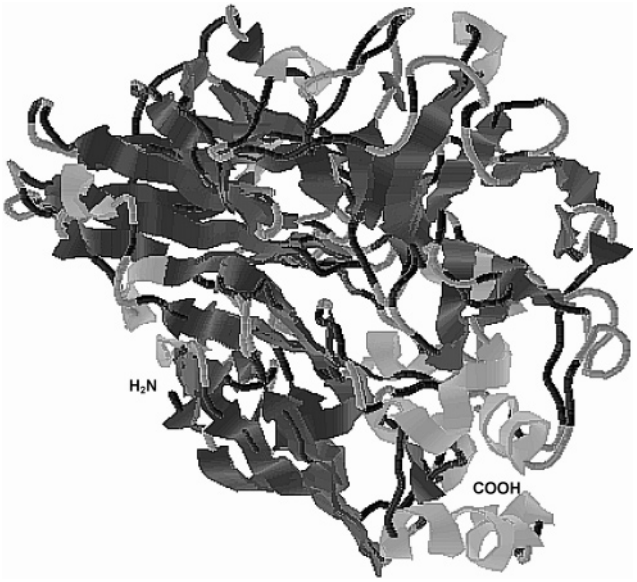

Figure 6. Ribbon presentation of the *P. sapidus* laccase (accession number AJ786026) generated with SWISS-MODEL (*31*).

can also serve as a baking aid, as the rheological properties of dough can be controlled via laccase-catalyzed oxidative cross-linking of arabinoxylans (*39*, *40*). Furthermore, arabinoxylans can be coupled enzymatically to protein fractions of wheat gluten, thus modifying dough rheology (*41*). Brinch and Pedersen subjected a *Polyporus pinsitus* laccase expressed in *Aspergillus oryzae* to a series of toxicological tests and documented its safety in use for the food-processing industry (*42*).

Further studies on the genome as well as on enzyme inducibility by lignified polymers will be necessary to fully disclose the secrets of the *P. sapidus* laccase family. The reusability of the immobilized fungal mycelium, the high activity levels, and the progressing work on genetically engineered basidiomycetes (*43*) hold much promise for upscaling and biotechnological production of laccases using renewable substrates.

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