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ORIGINAL PAPER

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Purification and characterisation of a novel laccase from the ascomycete *Melanocarpus albomyces*

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Abstract A novel laccase from the ascomycete *Melanocarpus albomyces* was purified and characterised. The enzyme was purified using anion exchange chromatography, hydrophobic interaction chromatography and gel filtration, and the purified laccase was biochemically characterised. It had activity towards typical substrates of laccases including 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate), dimethoxyphenol, guaiacol, and syringaldazine. The laccase showed good thermostability and it had a pH optimum at neutral pH, both unusual properties for most known fungal laccases. The activity of the laccase from *M. albomyces* was highest at 60–70°C. With guaiacol and syringaldazine the pH optima were rather broad: 5–7.5 and 6–7, respectively. It retained 50% of its activity after 5 h incubation at 60°C. The molecular weight of the laccase was about 80 kDa and the isoelectric point 4.0. The ultraviolet-visible absorption and electron paramagnetic resonance spectra of the purified laccase indicated that the typical three types of copper were present.

Introduction

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are multi-copper enzymes belonging to the group of blue oxidases. They catalyse oxidation of a surprisingly wide variety of organic and inorganic compounds, including diphenols, polyphenols, diamines, substituted phenols, and aromatic amines by a one-electron transfer mechanism. Molecular oxygen is used as the electron acceptor. The substrate loses a single electron and usually forms a free radical. The unstable radical may undergo further laccase-catalysed oxidation or non-enzymatic reactions including hydration, disproportionation, and polymerisation (Thurston 1994).

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Laccase or laccase-like activity has been demonstrated in higher plants, some insects and a few bacteria (Gianfreda et al. 1999). However, most known laccases are of fungal origin, especially from the class of white-rot fungi. Laccases are involved in several physiological functions, such as lignin biosynthesis, plant pathogenesis, insect sclerotisation, and degradation of lignocellulosic materials. It is well recognised that laccases are involved in both polymerisation and depolymerisation processes of lignin (Thurston 1994). Laccases are seen as industrially interesting enzymes because they have shown potential in various applications including pulp bleaching, textile dye bleaching, detergents, and enzymatic conversion of chemical intermediates (Xu 1999).

For many applications it is important to find an enzyme that is thermostable and works at relatively high pH-values. Thermophilic fungi could comprise a potential source of thermostable enzymes. A thermophilic ascomycete *Melanocarpus albomyces*, formerly known as *Myriococcum albomyces*, has previously been reported to produce xylanases and cellulases with pronounced thermal stability and activity at alkaline pH range (Jain et al. 1998; Prabhu and Maheshwari 1999). We report here that *M. albomyces* also produces a laccase with very interesting pH and temperature characteristics.

Materials and methods

Fungal strain

Melanocarpus albomyces (VTT D-96490, originally isolated from soil in Saudi Arabia), was maintained on oatmeal agar (Difco, Detroit, Mich.).

Media and culture conditions

M. albomyces was cultivated on liquid medium containing (per litre) 25 g glucose, 27.5 g yeast extract, 0.5 mg Indulin AT (Sigma, St. Louis, Mo.) and 0.04 l mineral stock solution containing (per litre) 1.0 g $CaCl_2 \cdot 2H_2O$, 1.0 g $FeSO_4 \cdot 7H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.16 g $CuSO_4 \cdot 5H_2O$, and 1.0 g Na_2EDTA. Glucose was autoclaved separately. First, 100 ml of medium was inoculat-

ed with agar pieces cut from well-grown mycelium on oatmeal agar. After 2 days cultivation at 37°C (160 rpm), the culture was homogenized and used to inoculate 1 l of culture medium in a 3 l Erlenmeyer flask. The flasks were incubated at 37°C on a rotary shaker (160 rpm). Laccase activity in the culture filtrate was measured daily and the enzyme was collected when laccase activity reached its maximum. The mycelium was removed by filtration through Whatman no. 1 filter paper.

Protein and enzyme activity determinations

Laccase activity was measured according to Niku-Paavola et al. (1988) using ABTS [2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate)], (Boehringer Mannheim; Mannheim, Germany) as a substrate. The laccase activity was also measured with syringaldazine (Sigma) according to Leonowicz and Grzywnowicz (1981), guaiacol (Sigma) according to Paszczynski et al. (1985), and 2,6-dimethoxyphenol (Sigma) according to Wariishi et al. (1992). All these activity assays were carried out in 25 mM succinate buffer (pH 4.5) at 25°C using a two-beam spectrophotometer (Lambda 20, Perkin-Elmer, Überlingen, Germany). The activity towards tyrosine was measured at a tyrosine concentration of 0.2 mM in 50 mM sodium phosphate buffer (pH 7) at 25°C and at 475 nm by monitoring dopachrome formation and using the molar extinction coefficient ε 3,600 M^{-1} cm^{-1} (Lerch and Ettlinger 1972). Activities were expressed as nanokatals. The protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad, Richmond, Calif.) with bovine serum albumin as a standard.

Protein purification

The clear culture supernatant was concentrated and the buffer changed to 10 mM acetate buffer, pH 5, with an Amicon 8400 ultrafiltration unit using a PM30 membrane (Millipore, Bedford, Mass.). The subsequent purification steps were carried out at room temperature. The sample was applied to a DEAE Sepharose Fast Flow column (1.6×13 cm; Pharmacia, Uppsala, Sweden), which was pre-equilibrated with 10 mM acetate buffer, pH 5. Proteins were eluted with a linear 0–200 mM Na_2SO_4 gradient (90 ml) in acetate buffer. Laccase-positive fractions were pooled. Using Na_2SO_4, the pH and ionic strength of the pooled sample were adjusted to correspond to those of 400 mM Na_2SO_4 in 20 mM citrate buffer, pH 5. The sample was applied to a Phenyl Sepharose Fast Flow column (1.6×9 cm; Pharmacia) pre-equilibrated with 20 mM citrate buffer, pH 5, containing 400 mM Na_2SO_4. Proteins were eluted with a linear 400–0 mM Na_2SO_4 gradient (90 ml) in citrate buffer, thereafter with 2 mM citrate buffer (pH 5), and finally with distilled water. Laccase-positive fractions were pooled, concentrated, and applied to a Sephacryl S-100 HR column (1.6×90 cm; Pharmacia) equilibrated with 100 mM sodium phosphate buffer, pH 7. Active fractions were pooled and concentrated on an Amicon PM10 membrane (Millipore).

SDS-PAGE (12% Tris-HCl Ready Gel, Bio-Rad) was performed according to Laemmli (1970). Protein bands were visualised by staining with Coomassie Brilliant Blue (R350; Pharmacia) and compared with molecular weight markers (Prestained Protein Marker Broad Range Cat. no. 7708S; New England Biolabs, Beverly, Mass.).

Determination of isoelectric point

The isoelectric point of *M. albomyces* laccase was determined by isoelectric focusing within the pH range of 2.5–5.0 (Pharmalyte 2.5–5.0 for IEF, Pharmacia) on an LKB 2117 Multiphor II Electrophoresis System (LKB Pharmacia, Bromma, Sweden) according to the manufacturer's instructions. Bands containing laccase activity were visualised by staining the gel with 2 mM ABTS in 25 mM succinate buffer (pH 4.5) and proteins by Coomassie Blue staining.

Enzyme activity and stability with respect to pH and temperature

The pH optimum for purified *M. albomyces* laccase was determined in McIlvaine (Dawson et al. 1959) buffer within a pH range of 2.2–8.0 using ABTS, guaiacol and syringaldazine as substrates. The stability of the enzyme at different pH-values was determined in McIlvaine buffer by incubating the purified enzyme solution (20 nkat/ml) at different pH-values at room temperature. The residual laccase activity was determined by measuring the activity of the enzyme solutions with ABTS as described above. The effect of temperature on enzyme activity was determined by measuring the enzyme activity with ABTS and guaiacol within a temperature range of 25–80°C. With ABTS the measurements were performed in 25 mM succinate buffer at pH 4.5, and with guaiacol in 50 mM citrate buffer at pH 6. The temperature stability was determined by incubating the enzyme solution (200 nkat/ml) in 60 mM citrate buffer (pH 6) in different temperatures and determining the residual enzyme activity with guaiacol.

Spectra

The optical absorption spectrum of purified *M. albomyces* laccase was measured with a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan). Electron paramagnetic resonance (EPR) spectra were recorded with a Bruker ESP 300 X-band spectrometer (Bruker, Karlsruhe, Germany) at 9.44 GHz and 12 K (cryostat from Oxford Instruments, Oxford, UK). Modulation frequency was 100 kHz, modulation amplitude 0.99 mT, sweep time/scan 168 s, sweep width 0.10 T, microwave power 1.00 mW, and protein concentration 50 µM.

Inhibition of laccase activity

The effect of various inhibitors on laccase activity was determined by measuring the oxygen consumption during the enzyme reaction with ABTS in sealed and fully filled Erlenmeyer flasks with an Orion Research 081010 oxygen electrode (software: SensorLink PCM800; Orion, Espoo, Finland). The oxygen consumption rates (nmol l^{-1} s^{-1}) were measured from solutions containing 15 µg of purified laccase (corresponding to an activity of about 15 nkat), 2 mM ABTS, and 0–5 mM of various inhibitors (added simultaneously with ABTS) in 50 mM citrate buffer (pH 5) in a 30 ml reaction volume. All measurements were taken in triplicate.

N-Terminal amino acid sequencing

The N-terminus of the protein and internal peptides were sequenced according to Edman degradation chemistry using PE Biosystems Procise Sequencer (PE Biosystems, Foster City, Calif.). For peptide preparation, the lyophilised protein was reduced with dithiothreitol, carboxymethylated with iodoacetamide and cleaved with sequencing grade trypsin (Promega, Madison, Wisc.) at an enzyme/substrate mass ratio of 1:100 for 12 h at 37°C in 0.1 M ammonium bicarbonate, pH 8.3 (Stone et al. 1988). The peptides generated were separated by reversed-phase high performance liquid chromatography (HP 1050, HP GmbH, Waldbronn, Germany; Vydac C-18 column, Grace Vydac, Hesperia, Calif.) with a linear acetonitrile gradient (0–60% acetonitrile in 0.1% trifluoroacetic acid). The amino acid sequence comparison of *M. albomyces* laccase with other laccase sequences was carried out with SIM-Alignment tool for protein sequences (Huang and Miller 1991).

Results

Enzyme purification

Laccase-positive fractions eluted as a single peak at about 100 mM salt concentration in anion exchange

Table 1 Purification of extracellular laccase from *Melanocarpus albomyces*

Purification step	Activity (nkat/ml)	Protein (mg/ml)	Volume (ml)	Specific activity (nkat/mg)	Activity yield (%)	Purification factor
Culture filtrate	23	5.9	250	3.9	100	1
Concentrated ultrafiltrate	35	3.4	170	10.2	103	2.6
Anion exchange chromatography	166	1.3	29	128	84	33
Hydrophobic interaction column	1,600	1.8	1.2	889	33	228
Gel filtration	1,250	1.1	0.8	1,136	17	292

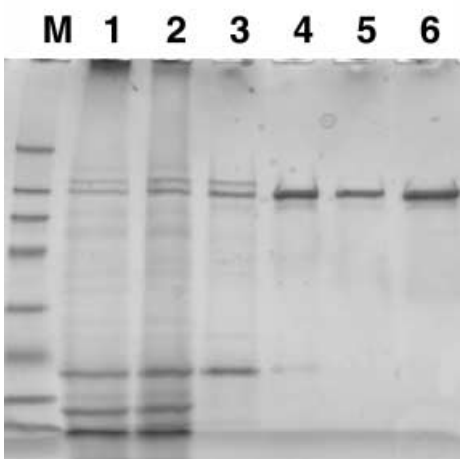

Fig. 1 SDS-PAGE of *Melanocarpus albomyces* laccase after different purification steps. Lanes: *M* Molecular weight marker (175, 83, 62, 47.5, 32.5, 25, 16.5, 6.5 kDa), *1* culture supernatant (118 µg protein), *2* concentrated ultrafiltrate (68 µg), *3* pooled laccase fractions after DEAE Sepharose (9 µg), *4* pooled laccase fractions from phenyl Sepharose (4 µg), *5* purified *M. albomyces* laccase (2 µg), *6* purified *M. albomyces* laccase (4 µg)

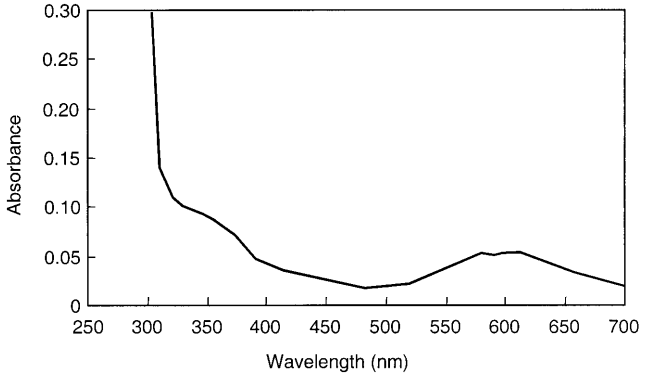

Fig. 2 The ultraviolet-visible absorption spectrum of *M. albomyces* laccase

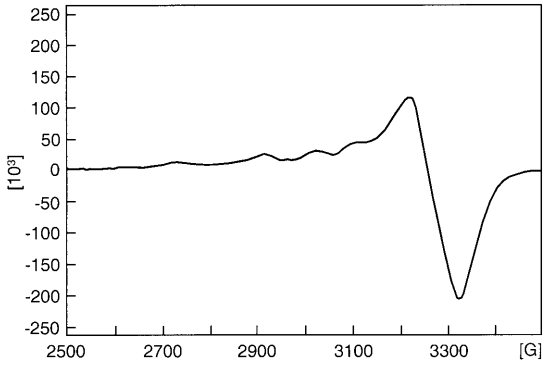

Fig. 3 The electron paramagnetic resonance (EPR) spectrum of *M. albomyces* laccase

chromatography. Laccase remained in the hydrophobic interaction column during decreasing salt gradient and eluted as a single peak with 2 mM citrate buffer. An overall 292-fold purification and activity recovery of 17% was achieved. Table 1 shows a summary of the purification. The purified laccase showed apparent homogeneity on SDS-PAGE (Fig. 1.). The molecular mass of the laccase as determined by SDS-PAGE was 80 kDa. Under nondenaturing conditions in isoelectric focusing both the culture filtrate and the purified laccase showed only one major band at pH 4.0 when stained with ABTS (results not shown).

Spectra

The purified laccase had a blue colour typical of copper-containing proteins. The ultraviolet-visible absorption spectrum of the laccase showed two peaks at 280 and 600 nm and a shoulder at 330 nm as shown in Fig. 2. The peak at 600 nm is typical for the type I Cu(II), and the shoulder at 330 nm suggests the presence of the type III binuclear Cu(II) pair (Eggert et al. 1996). Figure 3 presents the EPR absorption spectrum of purified *M. albomyces* laccase. The spectrum is typical of fungal laccases and it reveals the presence of type II (hyperfine line above 2,700 G) and type I (region 2,900–3,200 G) Cu(II) ions (Karhunen et al. 1990). The estimated parameters (g_{II} and A_{II}) are 2.18 and 0.010 cm^{-1} for the narrowly spaced signal and 2.25 and ≥0.019 cm^{-1} for the widely spaced signal.

Laccase activity with respect to pH and temperature

The pH optima for the purified laccase were determined with ABTS, guaiacol, and syringaldazine as substrates. The results are shown in Fig. 4. The lowest pH optimum, 3.5, was determined in oxidizing ABTS. Oxidation of guaiacol showed a very broad pH optimum from 5.0 to 7.5. The pH optimum for syringaldazine was within the

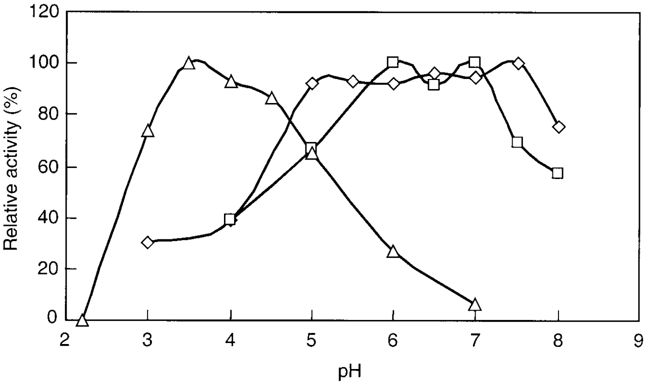

Fig. 4 pH activity profiles of purified *M. albomyces* laccase with 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) (*triangles*), guaiacol (*diamonds*) and syringaldazine (*squares*) as substrates

N-terminus:

MaL	E P T C N T P S N R A C W S D G - F D I N T D Y E V S T P
NcL 55	G G G C N S P T N R Q C W S P G - F N I N T D Y E L G T P
PaL 46	Q S S C H T A A N R A C W A P G - F D I N T D Y E V S T P
CpL 33	Q P N C N T A S N R A C W I S G S Y D I T T D Y E V K T P

1st internal peptide:

MaL	D I D L G V F P I T D Y Y Y R
NcL 215	D V D L G P F P L T D Y Y Y D
PaL 209	D I D L G V F P I T D Y Y H K
CpL 197	D I D L G P L V L S D Y Y Y K

2nd internal peptide:

MaL	F V F D P A V D L A R
NcL 503	Y V F D P A V D M A R
PaL 503	Y R F N P A T D M A L
CpL no significant similarity	

Fig. 5 Comparison of the N-terminal amino acid sequence and two internal peptide sequences from *M. albomyces* laccase (*MaL*) with *Neurospora crassa* (*NcL*) (Germann et al. 1988), *Podospora anserina* (*PaL*) (Fernández-Larrea and Stahl 1996), and *Cryphonectria parasitica* (*CpL*) (Choi et al. 1992) laccase sequences

Table 2 Specific activity of purified *M. albomyces* laccase towards different substrates. *ABTS* [2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate)], *2,6-DMP* 2,6-dimethoxyphenol

Substrate	Concentration (mM)	Specific activity (nkat/mg)
ABTS	5	836
Guaiacol	1.5	90
2,6-DMP	0.8	290
Syringaldazine	0.06	382
Tyrosine	0.2	0

Table 3 Effect of various inhibitors on oxidation of ABTS by purified *M. albomyces* laccase

Inhibitor	Concentration (mM)	Inhibition (%)
EDTA	1	1
	5	5
KCN	0.1	97
	0.5	99
	1	100
Kojic acid	1	0
L-Cysteine	1	0
NaCl	1	0
NaF	0.5	38
	1	48
NaN_3	0.5	97
	1	99
p-Coumaric acid	0.5	0
	1	0
SDS	1	0

range of 6–7. Interestingly, the laccase still showed relatively high activity at pH 8, both with syringaldazine and guaiacol (60 and 75%, respectively). The purified laccase remained quite stable within the pH range of 4–8 after 22 h incubation. However, at pH-values lower than 4, the enzyme lost its activity totally.

The activity of purified *M. albomyces* laccase was determined at various temperatures (25–90°C) using ABTS and guaiacol as substrates. The enzyme showed highest activity at 60–70°C. Beyond 70°C the activity dropped sharply. The activity remained unaltered after prolonged incubation (over 100 h) at 40°C, whereas it showed a half-life of about 50 h, 5 h, and 12 min at 50, 60, and 70°C, respectively.

Substrates and inhibitors

Table 2 presents the specific activities of purified *M. albomyces* laccase with various substrates. The specific activity was highest with ABTS. The other typical substrates for laccases, 2,6-dimethoxyphenol, syringaldazine, and guaiacol, were also oxidized by this enzyme. However, tyrosine was clearly not a substrate, thus this laccase does not belong to the group of tyrosinase-type polyphenol oxidases (EC 1.10.3.1; EC 1.14.18.1). The inhibition of laccase activity with different inhibitors is shown in Table 3. Sodium azide and potassium cyanide were very effective inhibitors of *M. albomyces* laccase. At a concentration of 1 mM, potassium cyanide inhibited totally and sodium azide inhibited 99% of the laccase activity. Sodium fluoride and EDTA caused some inactivation, but sodium chloride, *p*-coumaric acid and SDS did not have any inhibition effect on this laccase under the test conditions.

N-Terminal amino acid sequence

The N-terminal and two internal amino acid sequences of *M. albomyces* laccase were determined. When compared to the amino acid sequences of known laccases, the highest identity was found with ascomycete laccases (Fig. 5), namely *Neurospora crassa* (Germann et al. 1988), *Podospora anserina* (Fernández-Larrea and Stahl 1996), and *Cryphonectria parasitica* (Choi et al. 1992). The overall identity based on these sequences was highest with the laccases of *N. crassa* and *P. anserina* (70%). With basidiomycete laccases, e.g. *Trametes versicolor*

(Ong et al. 1997), *Phlebia radiata* (Saloheimo et al. 1991), *Pleurotus ostreatus* (Giardina et al. 1995), and *Rhizoctonia solani* (Wahleithner et al. 1996), the identity was found to be low (below 30%). The amino acid identity between *M. albomyces* laccase and the laccase from the ascomycete *Aspergillus nidulans* (Aramayo and Timberlake 1990) was also remarkably low (below 20%).

Discussion

M. albomyces has previously been reported to produce several thermostable and alkaline xylanases, as well as cellulases (Vehmaanperä et al. 1997; Jain et al. 1998; Prabhu and Maheshwari 1999). According to Jain et al. (1998), *M. albomyces* xylanase has its maximum activity at 70°C and is stable at that temperature for more than 2 h. In addition, the xylanase retains more than 50% of its activity at pH 10. The results reported here are the first to indicate that this thermophilic fungus also produces a thermostable laccase.

The enzyme purified from *M. albomyces* was able to oxidize typical substrates for laccases: a variety of phenolic compounds and non-phenolic ABTS. As previously reported, many fungal laccases show highest activity towards ABTS. The relative activities towards ABTS, guaiacol, and 2,6-dimethoxyphenol for *Pycnoporus cinnabarinus* laccase were 100, 31, and 22%, respectively, for *Coriolus hirsutus* laccase, 100, 40, and 39% respectively (Eggert et al. 1996) and for *Trichophyton rubrum* laccase 100, 33, and 36%, respectively (Jung et al. 2002). The *M. albomyces* laccase also had highest activity towards ABTS followed by syringaldazine, 2,6-dimethoxyphenol, and guaiacol. Similar to other laccases the enzyme did not show tyrosinase activity (Thurston 1994). The UV-visible absorption spectrum of the purified enzyme from *M. albomyces* showed a shoulder at 330 nm, which corresponds to a type-3 binuclear copper and a peak at 600 nm corresponding to a type-1 or the blue copper atom. These characteristics, as well as the EPR absorption spectrum, indicate that all three types of copper atoms are present (Eggert et al. 1996; Xu et al. 1996). Based on the substrate specificity and spectral data we conclude that the enzyme purified from *M. albomyces* is a true laccase.

Many sulfhydryl-containing compounds, e.g. L-cysteine, are often referred to as laccase inhibitors. However, Johannes and Majcherczyk (2000) recently showed that the observed inhibitory effect is actually caused by the reduction of the oxidized substrate by the sulfhydryl compounds and not by true inhibition of the enzyme. That is why we tested the inhibitors by monitoring oxygen consumption in the reaction and not spectrophotometrically. From our results it is obvious that L-cysteine, often considered a substrate for laccases, did not inhibit the oxygen consumption by laccase. *M. albomyces* laccase was strongly inhibited by the typical laccase inhibitors potassium cyanide, sodium azide and sodium fluoride, but it was not sensitive to EDTA, SDS, coumaric acid or kojic acid.

The isoelectric focusing PAGE indicated the presence of only one isoform of laccase in the culture supernatant, whereas many laccases have been reported to be produced as multiple isoforms, e.g. *Trametes villosa* produces at least three laccase isoforms (Bourbonnais et al. 1995) and *Pleurotus ostreatus* four isoforms (Palmieri et al. 2000). The molecular weight of the purified laccase (ca. 80,000 Da) is consistent with the molecular weights of most other fungal laccases, which have been reported to be between 60,000 and 80,000 Da (Thurston 1994).

The pH optimum of the *M. albomyces* laccase depended very much on the substrate. The laccase exhibited a rather low pH optimum (3.5) with ABTS as a substrate, like many other fungal laccases (Xu 1997; Robles et al. 2000). With guaiacol and syringaldazine, the pH optima were very broad: 5–7.5 and 6–7, respectively. The difference in pH optima for ABTS and phenolic substrates is typical for laccases and it reflects the difference in oxidation mechanism with different substrates. Since the oxidation of ABTS does not involve protons, the only effect of elevated pH on oxidation rate is the increasing inhibition of laccase by OH^- ions (Xu 1997). The instability of the oxidation product of syringaldazine at pH-values above 7 may also affect the activity results obtained at alkaline pH-values. Compared to many other fungal laccases, e.g. those of *Trametes versicolor* (Schlosser et al. 1997), *Pycnoporus cinnabarinus* (Eggert et al. 1996), *Trametes villosa* (Xu 1997), and *Thermoascus aurantiacus* (Machuca et al. 1998), the noteworthy feature of the *M. albomyces* laccase is its activity at alkaline pH: the enzyme still showed 75% of maximum activity at pH 8 with guaiacol. In addition, the enzyme showed good stability at alkaline pH-values, over 90% of the activity remained after 22 h incubation at pH 8.

Besides high stability at elevated pH-values, the *M. albomyces* laccase showed good thermostability. Its activity remained virtually unaltered at 60°C for up to 2 h. This kind of combination of thermal- and pH-stability is very rare among fungal laccases. Many other laccases that have a pH optimum around 6–7 are not as thermostable as *M. albomyces* laccase. For example, laccases from *Coprinus friesii*, *Panaeolus papilionaceus* and *Panaeolus sphinctrinus* have unusually high pH optima (7–8) with dimethoxyphenol, but do not remain active at 60°C (Heinzkill et al. 1998). Similarly, the neutral laccases from *Myceliophthora thermophila* (Berka et al. 1997), *Chaetomium thermophilum* (Chefetz et al. 1998), and *Coprinus cinereus* (Schneider et al. 1999) retain less than 75% of maximum activity at 60°C after 1 h. In addition, thermal activation in which incubation at higher temperatures results in higher activity was clearly observed with *M. albomyces* laccase, as previously reported for laccases from *M. thermophila* and *S. thermophilum* (Xu et al. 1996).

The comparison of the N-terminal amino acid sequence and two internal peptide sequences of *M. al-*

bomyces laccase with other fungal laccases showed that the *M. albomyces* laccase clearly resembles other ascomycete laccases. In comparison, the identity with basidiomycete laccases was low. These results support the theory that the genes of ascomycete and basidiomycete laccases have evolved significantly after the phylogenetic divergence of the two classes of fungi (Fernández-Larrea and Stahl 1996; Berka et al. 1997). Currently, we are isolating the gene encoding the *M. albomyces* laccase in order to express the protein heterologously to achieve higher production levels as well as to further analyse the enzyme properties.

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