Single electron transfer by an extracellular laccase from the white-rot fungus *Pleurotus ostreatus*

Hong-Duk Youn, Kyu-Jung Kim,[†] Jin-Soo Maeng, Young-Hoon Han, In-Beom Jeong, Gajin Jeong, Sa-Ouk Kang and Yung Chil Hah

Author for correspondence: Sa-Ouk Kang. Tel: +82 2 880 6703. Fax: +82 2 888 4911.
e-mail: kangsaou@alliant.snu.ac.kr

Department of Microbiology, College of Natural Sciences, and Research Center for Molecular Microbiology, Seoul National University, Seoul 151-742, Republic of Korea

Two different bands with laccase activity were obtained after nondenaturing PAGE of the culture filtrate of *Pleurotus ostreatus*. Immunoblot analysis revealed that antisera raised against laccase I were not reactive to laccase II. Laccase I, which exhibited faster mobility on nondenaturing polyacrylamide gel, was purified 42.9-fold with an overall yield of 10.8%. Gel filtration and SDS-PAGE revealed that laccase I is a single polypeptide with a molecular mass of approximately 64 kDa. Laccase I contained 12.5% carbohydrate by weight and 3.9 mol copper (mol protein)⁻¹. The absorption spectrum of laccase I showed a type 1 signal at 605 nm and EPR spectra showed that the parameters of the type 1 and type 2 Cu signals were $g_{\parallel} = 2.197$ and $A_{\parallel} = 0.009 \text{ cm}^{-1}$, and $g_{\perp} = 2.263$ and $A_{\perp} = 0.0176 \text{ cm}^{-1}$, respectively. The data obtained from the pH profiles suggested that two ionization groups, whose pK_a values were 5.60–5.70 and 6.70–6.85, may play an important role in the active site of laccase I as the ligand of copper metal. The optimal pH and temperature for the activity of laccase I were 6.0–6.5 and 30–35 °C, respectively. The enzyme had affinity for various lignin-related phenolic compounds: the K_m values for ferulic acid and syringic acid were 48 and 89 μM , respectively. EPR spectroscopic study of the action of laccase I on 3,5-dimethoxy-5-hydroxyacetophenone indicated that this enzyme catalyses single electron transfer with the formation of the phenoxy radical as an intermediate.

Keywords: *Pleurotus ostreatus*, laccase, single electron transfer, phenoxy radical

INTRODUCTION

Lignin is a structurally amorphous, aromatic biopolymer found abundantly in woody plants and extremely resistant to microbial attack in nature, but white-rot fungi are able to degrade it efficiently. Lignin degradation by white-rot basidiomycetes is considered to be a nonspecific, oxidative process achieved by the action of either peroxidase, which depends on hydrogen peroxide, or laccase, which depends on oxygen molecules (Kirk & Farrell, 1987). The ability of fungal laccases to oxidize lignin-related phenolic compounds suggests that these enzymes play a role in lignin degradation (Eriksson *et al.*, 1990). Laccases have been isolated from various fungi and their physiological functions have been discussed (Froehner & Eriksson, 1974; Wood, 1980; De Vries *et al.*, 1986; Niku-Paavola *et*

al., 1988; Rehman & Thurston, 1992; Jeong *et al.*, 1992). The white-rot fungus *Pleurotus ostreatus* is a well-known lignin-degrader (Agosin *et al.*, 1985) and it can produce various enzymes, such as extracellular peroxidase (Kang *et al.*, 1993), veratryl alcohol oxidase (Sannia *et al.*, 1991), glucose oxidase (Shin *et al.*, 1993) and laccase (Sannia *et al.*, 1986; Kim *et al.*, 1987), all of which are related to lignin degradation.

In the present paper, we report the further physico-chemical properties of one of two immunologically different laccases from the culture filtrate of *P. ostreatus*.

METHODS

Micro-organism and growth conditions. *P. ostreatus* NFFA 4501, obtained from the Korean Forest Research Laboratory, was used as the source of laccase I for purification. The organism was cultured in shaken, 500 ml Erlenmeyer flasks containing 200 ml complex medium [1% (w/v) glucose, 0.5%

[†] Present address: Department of Biology, College of Sciences, Kangreung National University, Republic of Korea.

peptone, 0.5% yeast extract, 1% (w/v) malt extract] at 28 °C for 6 d. For the induction of the enzyme, ferulic acid was added to a final concentration of 1 mM after 2 d culture (Kim *et al.*, 1986).

Chemicals. DEAE Sephadex A-50, Sephacryl S-200HR and syringaldazine were purchased from Sigma, ascorbate oxidase and molecular mass markers for gel filtration chromatography and SDS-PAGE from Boehringer Mannheim, and Protein-Pak DEAE 5PW column from Waters. All other reagents used were of the highest quality generally available.

Enzyme assays. Laccase activity was determined by measuring the increase in A_{530} using syringaldazine as substrate at room temperature (Leonowicz & Grzywnowicz, 1981). One unit of enzyme activity was defined as the amount of enzyme producing 1 μ mol reaction product min^{-1} . For kinetic studies, enzyme activity was determined by measurement of oxygen consumption with a Clark oxygen electrode (Yellow Springs Instrument). After nondenaturing PAGE, laccase activity was visualized by incubation with 50 μ M syringaldazine in 50 mM sodium acetate buffer (pH 5.5) and fixing with 10% (v/v) glacial acetic acid.

Enzyme purification. All purification steps were performed at 4 °C. The culture suspension used for enzyme purification was filtered through Whatman no. 1 filter paper. Solid ammonium sulphate was added to the supernatant to 80% saturation. The precipitate was collected by centrifugation and dissolved in 20 mM sodium phosphate buffer (pH 7.0). Protamine sulphate was added to a final concentration of 1% (w/v) to remove brownish pigments. The precipitate was removed by centrifugation and the supernatant was dialysed overnight against the same buffer. The dialysed protein was loaded onto a DEAE Sephadex A-50 column (4 $\times$ 40 cm) equilibrated with the same buffer. The column was then washed and the protein eluted using a linear concentration gradient of 0–0.8 M NaCl in the same buffer. Two separate fractions with laccase activity were combined and concentrated using an Amicon YM10 membrane. The concentrated sample was applied to a Sephacryl S-200HR column (2.8 $\times$ 120 cm) equilibrated with 20 mM sodium phosphate buffer (pH 6.0). Active fractions were again pooled and concentrated using a YM10 membrane. The concentrated protein was further purified using a Waters Delta Prep 4000 chromatography system. The sample was loaded onto a Protein-Pak DEAE 5PW column (2.15 $\times$ 15 cm) equilibrated with 20 mM sodium phosphate buffer (pH 6.0). The column was washed with the same buffer and laccase I and II were eluted using a linear concentration gradient of 0.0–0.4 M NaCl at a flow rate of 5 ml min^{-1} .

Preparation of anti-laccase I antisera. Purified laccase I was subjected to SDS-PAGE. The polypeptide band was cut from the gel and pulverized with a homogenizer. This preparation was used as an immunogen. Each of three mice was initially injected subcutaneously with approximately 15 μ g laccase I emulsified in Freund's complete adjuvant. Subsequently, approximately 15 μ g laccase I was injected at 2-week intervals for 8 weeks.

Immunoblot analysis. SDS-PAGE was performed on a 10% (w/v) polyacrylamide gel. Electrophoretic transfer of proteins was carried out according to the method of Towbin *et al.* (1979). The proteins were electrotransferred to a nitrocellulose membrane for 1 h at 170 mA in a Bio-Rad transblot apparatus. Three lanes, containing molecular mass markers, laccase I and II, were cut out and stained with 0.1% amidoblack and destained until the bands were discernible. The rest of the membrane was blocked by incubation with 0.5% BSA in TBST [10 mM Tris (pH 8.0), 150 mM NaCl, 0.05% Tween 20] for 30 min at room temperature. The membrane was incubated in TBST containing anti-laccase I antiserum at 1:2000 dilution overnight and then washed with TBST for 30 min. It was incubated again in TBST

containing anti-mouse IgG (whole molecule) alkaline phosphatase conjugate at 1:10000 dilution for 90 min, washed with TBST for 30 min and developed with nitro blue tetrazolium plus 5-bromo-4-chloro-3-indolyl phosphate in alkaline phosphatase buffer [100 mM Tris (pH 9.5), 100 mM NaCl, 5 mM MgCl_2].

Molecular mass determination. The molecular mass of the native enzyme was determined by gel filtration chromatography on a Sephacryl S-200 HR column. The column was calibrated with aldolase (158 kDa), albumin (68 kDa), chymotrypsinogen (25 kDa), and cytochrome *c* (12.5 kDa). SDS-PAGE was performed on a 7.5% polyacrylamide gel (Laemmli, 1970). As standard markers, β -galactosidase (116.4 kDa), fructose-6-phosphate kinase (85.2 kDa), glutamate dehydrogenase (55.6 kDa), aldolase (39.2 kDa), and triosephosphate isomerase (26.6 kDa) were used.

Determination of the concentrations of protein, carbohydrate and copper. The protein concentration was estimated by the Lowry method using BSA as the standard. The carbohydrate content was determined according to the method of Dubois *et al.* (1956) using glucose as the standard. The copper content was determined according to the method described by Brumby & Massey (1967).

Amino-terminal sequence analysis. The amino-terminal sequence of laccase I was determined with a MilliGen/Biosearch 6600 Prosequencer protein sequencing system (Millipore).

Spectroscopic studies. The absorption spectrum of native laccase I was determined in 50 mM sodium acetate buffer (pH 5.0) at room temperature using a Shimadzu model UV-265 spectrophotometer. EPR spectra of the native enzyme were recorded on a Bruker ESP 300S EPR spectrometer with modulation amplitude 2 mT, microwave power 20 mW and frequency 9.42 GHz. Probe temperature was regulated with a nitrogen cryostat equipped with a temperature control unit. Temperature was maintained at approximately 100 K. For the EPR spectroscopic detection of the phenoxy radical, the reaction mixture contained 0.1 M sodium phosphate buffer (pH 6.0), 0.5 U laccase I and 1.0 mM 3,5-dimethoxy-5-hydroxyacetophenone. The EPR spectrum of the phenoxy radical was measured at 323 K with modulation amplitude 50 μ T, microwave power 7.96 mW and frequency 9.43 GHz.

RESULTS AND DISCUSSION

Purification of laccases

Two different bands with laccase activity were visualized by nondenaturing PAGE of the culture filtrate of *P. ostreatus* (Fig. 1). Laccase I, which exhibited much faster mobility on nondenaturing polyacrylamide gel, appeared to be the major isozyme. Two isozymes with laccase activity were purified from the culture filtrate as summarized in Table 1. Laccase I was purified 42.9-fold with a recovery of 10.8% and laccase II was partially purified 23.9-fold with a recovery of 1.0%. The specific activities of purified laccases I and II were 3.00 and 1.67 U (mg protein^{-1}), respectively. The amount of laccase II obtained was insufficient for further purification.

Comparison of laccase I and laccase II

Laccases I and II had distinct mobilities in nondenaturing PAGE (see above). The two laccases were also immunologically different (Fig. 2b): antisera raised against laccase I recognized purified laccase I but not laccase II. However,

Fig. 1. Nondenaturing PAGE of two laccases from *P. ostreatus*. The polyacrylamide concentration was 10%. Laccase activity was detected by staining with 50 μ M syringaldazine in 50 mM sodium acetate buffer (pH 5.5) and fixing with 10% glacial acetic acid. Lanes: 1, purified laccase I (2 μ g); 2, partially purified laccase II (5 μ g).

the apparent molecular masses of these two enzymes were approximately equal in size as determined by 10% SDS-PAGE (approx. 64 kDa; Fig. 2a). The molecular mass of laccase I as determined by Sephacryl S-200 HR gel filtration chromatography, was similar to the value estimated by SDS-PAGE, indicating that the enzyme consists of a single polypeptide. Its molecular mass was slightly different from that of the laccase of *P. ostreatus* strain 3004 (approx. 59 kDa; Sannia *et al.*, 1986).

The amino-terminal sequence of laccase I was compared with those of other fungal laccases (Fig. 3). It was quite different from that of *Neurospora crassa* (Germann *et al.*, 1988), but similar to those of other white-rot fungi (Jeong *et al.*, 1992; Sannia *et al.*, 1986; Kosima *et al.*, 1990; Saloheimo *et al.*, 1991). It was however notable that the amino-terminal sequence of laccase I of *P. ostreatus* NFFA

Fig. 2. Electrotransfer of *P. ostreatus* laccases after 10% SDS-PAGE. (a) Protein stain with 0.1% amidoblack. Lanes: 1, molecular mass markers; 2, purified laccase I; 3, partially purified laccase II. (b) Immunoblot with anti-laccase I antiserum. Lanes: 4, purified laccase I; 5, partially purified laccase II.

<i>Pleurotus ostreatus</i> NFFA 4501	A I G P T G N M Y I V
<i>Pleurotus ostreatus</i> 3004	A I G S A G N T Y I V
<i>Coriolus hirsutus</i> IFO 4917	A I G P T A D L T I S
<i>Lentinus edodes</i> ATCC 48085	A I G P V T D L Q I V
<i>Phlebia radiata</i> ATCC 64658	S I G P V T N F H I V
<i>Neurospora crassa</i> FGSC 321	G G G G G C N S P T N

Fig. 3. Amino-terminal sequence comparison between laccase I of *P. ostreatus* NFFA 4501 and those of other fungi: *P. ostreatus* 3004 (Sannia *et al.*, 1986), *C. hirsutus* (Kosima *et al.*, 1990), *L. edodes* (Jeong *et al.*, 1992), *P. radiata* (Saloheimo *et al.*, 1991) and *N. crassa* (Germann *et al.*, 1988).

4501 was slightly different from that of laccase from *P. ostreatus* strain 3004 (Sannia *et al.*, 1986), suggesting that even laccases from the same fungal species may have

Table 1. Purification of extracellular laccases from *P. ostreatus*

Purification step	Total protein (mg)	Specific activity (U mg ⁻¹)	Recovery (%)	Purification (-fold)
Culture filtrate	760.0	0.07	100.0	1.0
Ammonium sulphate precipitation	81.0	0.33	54.0	4.7
Protamine sulphate precipitation	40.0	0.37	30.0	5.3
DEAE Sephadex A-50 chromatography	25.0	0.48	24.0	6.9
Sephacryl S-200HR chromatography	5.2	1.42	14.8	20.3
Protein-Pak DEAE 5PW chromatography				
Laccase I	1.8	3.00	10.8	42.9
Laccase II	0.3	1.67	1.0	23.9

Fig. 4. Absorption spectrum of *P. ostreatus* laccase I. Enzyme samples (1.4 mg) were dissolved in 50 mM sodium acetate buffer (pH 5.0). —, Native enzyme; ---, enzyme treated anaerobically with ascorbate. The inset shows the enlarged spectrum.

slightly different amino acid sequences which do not alter enzyme activity.

Laccase I was a glycoprotein containing 12.5% carbohydrate by weight. Most fungal laccases have been reported to contain 10–25% carbohydrate (Reinhammer, 1984).

Copper as a cofactor

Laccase I contained approximately 3.9 mol copper (mol protein)⁻¹ as determined by the method described by Brumby & Massey (1967). Ascorbate oxidase, which contains 8 mol copper (mol protein)⁻¹ (Mondovi & Avigliano, 1984), was estimated by this method to contain approximately 7.7 mol copper (mol protein)⁻¹. The absorption spectrum of laccase I had a type 1 signal at 605 nm, as expected from the blue colour (Fig. 4). The broad shoulder at 330 nm representing a type 3 Cu signal was not detected clearly in the absorption spectrum of native laccase. Anaerobic treatment with a reducing agent, ascorbic acid, led to a decrease in absorbance in this region, showing that type 3 Cu is present even though the signal is weak. *Phlebia radiata* laccase did not give the typical absorption maximum of type 3 Cu at 330 nm and contains pyrroloquinoline quinone (PQQ) instead of copper as a cofactor (Karhunen *et al.*, 1990). However, PQQ was not detected in *P. ostreatus* laccase using a hexanol extraction procedure (van der Meer *et al.*, 1988).

As in the spectrum of *Polyporus versicolor* laccase (Vännegård, 1972), the EPR spectra of laccase I showed a superposition of type 1 and type 2 signals (Fig. 5). The parameters of the type 1 Cu signal were $g_{\parallel} = 2.197$ and $A_{\parallel} = 0.009 \text{ cm}^{-1}$, and those of the type 3 Cu signal $g_{\parallel} = 2.263$ and $A_{\parallel} = 0.0176 \text{ cm}^{-1}$. These values closely resemble those of *Polyporus versicolor* (2.190 and

Fig. 5. X-band EPR spectra of *P. ostreatus* laccase I. Enzyme samples (3.8 mg) were dissolved in 50 mM sodium acetate buffer (pH 5.0) and scanned as described in Methods.

Fig. 6. pH profiles of the steady-state kinetic parameters k_{cat} and k_{cat}/K_m for the oxidation of syringaldazine by *P. ostreatus* laccase I. Reaction mixtures contained various concentrations of syringaldazine buffered with 0.1 M citrate/0.2 M disodium phosphate. Initial velocity was determined by measuring consumption of molecular oxygen.

0.0090 cm^{-1} , and 2.243 and 0.0194 cm^{-1} , respectively; Vännegård, 1972) and of *Phlebia radiata* (2.19 and 0.009 cm^{-1} , and 2.25 and 0.017 cm^{-1} , respectively; Karhunen *et al.*, 1990).

As judged from the colour, copper content, and absorption and EPR spectra, *P. ostratus* laccase I is a copper-containing protein like other fungal laccases.

Effects of temperature and pH on enzyme activity

The optimal temperature for laccase I activity under the standard assay conditions was 30–35 °C. The Arrhenius plot indicated an activation energy of 10.7 kJ mol^{-1} from 30–35 °C. The optimal pH for laccase activity was 6.0–6.5. As shown by the pH profiles of the steady-state kinetic parameters k_{cat} and k_{cat}/K_m for syringaldazine (Fig. 6), two ionization groups with pK_a values of 5.60–5.70 and

Table 2. Substrate specificity of laccase I from *P. ostreatus*

Reaction mixtures contained 0.1 mM substrate in 20 mM sodium phosphate buffer (pH 6.0). Initial velocity was measured after the addition of 0.01 U laccase I. Activity is expressed relative to that towards ferulic acid (45.4 nmol O₂ uptake (min)⁻¹), defined as 100%.

Substrate	Relative activity (%)
Ferulic acid	100.0
Syringic acid	76.2
Pyrogallol	45.7
Guaiacol	33.3
Vanillin	19.0
Gallic acid	6.2
Vanillic acid	4.8
<i>p</i> -Coumaric acid	4.3
Tyrosine	1.4
<i>trans</i> -Cinnamic acid	0.0
3,4-Dimethoxycinnamic acid	0.0
Veratryl alcohol	0.0

6.70–6.85 were observed. These two ionization groups may play an important role in the active site of laccase I as the ligand of copper metal.

Substrate specificity

Laccase I exhibited phenol oxidase activity towards various lignin-related compounds. As shown in Table 2, the enzyme had high affinity for phenolic compounds containing methoxyl and *p*-hydroxy groups directly attached to the benzene ring, but no affinity for non-phenolic compounds such as *trans*-cinnamic acid, 3,4-dimethoxycinnamic acid and veratryl alcohol. Substrate specificity was thus similar to that of *P. ostreatus* peroxidase (Kang *et al.*, 1993).

The K_m and k_{cat} for ferulic acid were 48 μ M and 4.10×10^3 nmol O₂ uptake min⁻¹ (nmol enzyme)⁻¹ and for syringic acid were 89 μ M and 2.54×10^3 nmol O₂ uptake min⁻¹ (nmol enzyme)⁻¹.

Single electron transfer

The EPR spectrum presented in Fig. 7 shows that 3,5-dimethoxy-5-hydroxyacetophenone, a lignin-related compound, was oxidized to a phenoxy radical by laccase I. The EPR parameters of the phenoxy radical were determined as follows: $a_{OCH_3} = 14.36$ mT (6 eq. protons), $a_{H(meta)} = 14.36$ mT (2 eq. protons) and $a_{CH_3} = 3.40$ mT (3 eq. protons). It was very similar to the EPR spectrum obtained from *Phanerochaete chrysosporium* lignin peroxidase except that the latter showed extra peaks due to a secondary radical signal (Odier *et al.*, 1988). The phenoxy radical from laccase I-catalysed oxidation of 3,5-dimethoxy-5-hydroxyacetophenone may be formed via

Fig. 7. EPR spectrum of the phenoxy radical produced by the action of laccase I on 3,5-dimethoxy-4-hydroxyacetophenone. The reaction conditions are described in Methods.

deprotonation of the *p*-hydroxy group attached to the benzene ring.

ACKNOWLEDGEMENTS

This work was supported by Korea Science and Engineering Foundation (KOSEF) research grant for SRC (Research Center for Molecular Microbiology, Seoul National University).

REFERENCES

- Agosin, E., Daudin, J. J. & Odier, E. (1985). Screening of white-rot fungi on [¹⁴C]lignin-labelled and [¹⁴C]whole-labelled wheat straw. *Appl Microbiol Biotechnol* **22**, 132–138.
- Brumby, P. E. & Massey, V. (1967). Copper determinations. *Methods Enzymol* **10**, 471–474.
- De Vries, O. M. H., Kooistra, W. H. C. F. & Wessels, J. G. H. (1986). Formation of an extracellular laccase by a *Schizophyllum commune* dikaryon. *J Gen Microbiol* **132**, 2817–2826.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Robers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substrates. *Anal Chem* **28**, 350–356.
- Eriksson, K.-E. L., Blanchette, R. A. & Ander, P. (1990). Biochemistry of lignin degradation. In *Microbial and Enzymatic Degradation of Wood and Wood Components*, pp. 253–307. Berlin: Springer-Verlag.
- Froehner, S. C. & Eriksson, K.-E. L. (1974). Purification and properties of *Neurospora crassa* laccase. *J Bacteriol* **120**, 458–465.
- Germann, U. A., Müller, G., Hunziker, P. E. & Lerch, K. (1988). Characterization of two allelic forms of *Neurospora crassa* laccase. *J Biol Chem* **263**, 885–896.
- Jeong, I.-B., Youn, H.-D., Maeng, J.-S., Kang, S.-O., Hah, Y. C., Jeong, G., Choi, H. T. & Kim, J.-H. (1992). Characterization of laccase excreted from *Lentinus edodes*. *Korean J Microbiol* **30**, 252–259.
- Kang, S.-O., Shin, K.-S., Han, Y.-H., Youn, H.-D. & Hah, Y. C. (1993). Purification and characterisation of an extracellular peroxidase from white-rot fungus *Pleurotus ostreatus*. *Biochim Biophys Acta* **1163**, 158–164.
- Karhunen, E., Niku-Paavola, M.-L., Viikari, L., Haltia, T., van der Meer, R. A. & Duine, J. A. (1990). A novel combination of

prosthetic groups in a fungal laccase: PQQ and two copper atoms. *FEBS Lett* **267**, 6–8.

Kim, K.-J., Shin, K.-S. & Hong, S.-W. (1986). Induction of extracellular polyphenol oxidase from two white-rot fungi. *Korean J Mycol* **14**, 43–47.

Kim, K.-J., Maeng, J.-S., Shin, K.-S., Kang, S.-O., Hah, Y. C. & Hong, S.-W. (1987). Characterization of laccase from *Pleurotus ostreatus*. *Korean J Microbiol* **25**, 148–156.

Kirk, T. K. & Farrell, R. L. (1987). Enzymatic combustion: the microbial degradation of lignin. *Annu Rev Microbiol* **41**, 465–505.

Kosima, Y., Tsukuda, Y., Kawai, Y., Tsukamoto, A., Sugiura, J., Sakaido, M. & Kita, Y. (1990). Cloning, sequence analysis, and expression of ligninolytic phenoloxidase genes of the white-rot basidiomycete *Coriolus hirsutus*. *J Biol Chem* **265**, 15224–15230.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Leonowicz, A. & Grzywnowicz, K. (1981). Quantitative estimation of laccase forms in some white-rot fungi using syringaldazine as a substrate. *Enzyme Microb Technol* **3**, 55–58.

van der Meer, R. A., Jongejan, J. A. & Duine, J. A. (1988). Identification and quantification of PQQ. In *PQQ and Quinoproteins*, pp. 111–122. Edited by J. A. Jongejan & J. A. Duine. Dordrecht: Kluwer Academic Publishers.

Mondovi, B. & Avigliano, L. (1984). Ascorbate oxidase. In *Copper Proteins and Copper Enzymes*, vol. III, pp. 101–118. Edited by R. Lontie. Boca Raton: CRC Press.

Niku-Paavola, M.-L., Karhunen, E., Salola, P. & Raunio, V. (1988). Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. *Biochem J* **254**, 877–884.

Odier, E., Mozuch, M. D., Kalyanaraman, B. & Kirk, T. K. (1988). Ligninase-mediated phenoxy radical formation and polymerization

unaffected by cellobiose:quinone oxidoreductase. *Biochimie* **70**, 847–852.

Rehman, A. U. & Thurston, C. F. (1992). Purification of laccase I from *Armillaria mellea*. *J Gen Microbiol* **138**, 1251–1257.

Reinhammar, B. (1984). Laccase. In *Copper Proteins and Copper Enzymes*, vol. III, pp. 1–35. Edited by R. Lontie. Boca Raton: CRC Press.

Saloheimo, M., Niku-Paavola, M.-L. & Knowles, J. K. C. (1991). Isolation and structural analysis of the laccase gene from the lignin-degrading fungus *Phlebia radiata*. *J Gen Microbiol* **137**, 1537–1544.

Sannia, G., Giardina, P., Luna, M., Rossi, M. & Buonocore, V. (1986). Laccase from *Pleurotus ostreatus*. *Biotechnol Lett* **8**, 797–800.

Sannia, G., Limongi, P., Cocca, E., Buonocore, F., Nitti, G. & Giardina, P. (1991). Purification and characterization of a veratryl alcohol oxidase enzyme from the lignin degrading basidiomycete *Pleurotus ostreatus*. *Biochim Biophys Acta* **1073**, 114–119.

Shin, K.-S., Youn, H.-D., Han, Y.-H., Kang, S.-O. & Hah, Y. C. (1993). Purification and characterisation of D-glucose oxidase from white-rot fungus *Pleurotus ostreatus*. *Eur J Biochem* **215**, 747–752.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**, 4350–4354.

Vänngård, T. (1972). Copper proteins. In *Biological Applications of Electron Spin Resonance*, pp. 411–447. Edited by H. M. Swartz, J. R. Bolton & D. C. Borg. New York: Wiley.

Wood, D. A. (1980). Production, purification and properties of extracellular laccase of *Agaricus bisporus*. *J Gen Microbiol* **117**, 327–338.

Received 11 August 1994; accepted 20 October 1994.