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

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 fungi 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 (Frohner & 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), veratral 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% sucrose, 2.5% agar].
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 $A_{580}$ using syringaldazine as substrate at room temperature (Leonowicz & Grzywnowicz, 1981). One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol 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 μM 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 the supernatant to 80 % saturation. 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 × 40 cm) equilibrated with the same buffer. The column was then washed and the protein eluted using a linear concentration gradient of 0-08 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 × 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 × 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 μg laccase I emulsified in Freund’s complete adjuvant. Subsequently, approximately 15 μg laccase I was injected at 2-week intervals for 8 weeks.

**Immunoblot analysis.** SDS-PAGE was performed on a 10 % (w/v) polyacrylamide gel. Electrophoresis of proteins was carried out according to the method of Towbin et al. (1979). The proteins were electrodried 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 % amido black 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 Brummy & Massey (1967).

**Amino-terminal sequence analysis.** The amino-terminal sequence of laccase I was determined with a Milligen/Biosearch 6600 Protein sequencer 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 phenox 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-hydroxycetophenone. The EPR spectrum of the phenox radical was measured at 323 K with modulation amplitude 50 μT, 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 10 %. 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,
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 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

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

![Fig. 2. Electropherogram of laccases from 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 antisera. Lanes: 4, purified laccase I; 5, partially purified laccase II.](image)

**Fig. 3.** Amino-terminal sequence comparison between laccase I of *P. ostreatus* NFFA 4501 and those of other fungi: *P. ostreatus* strain 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).

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Specific activity (U mg$^{-1}$)</th>
<th>Recovery (%)</th>
<th>Purification (-fold)</th>
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<tbody>
<tr>
<td>Culture filtrate</td>
<td>760.0</td>
<td>0.07</td>
<td>100.0</td>
<td>1.0</td>
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<tr>
<td>Ammonium sulphate precipitation</td>
<td>81.0</td>
<td>0.33</td>
<td>54.0</td>
<td>4.7</td>
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<td>Protamine sulphate precipitation</td>
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<td>0.37</td>
<td>30.0</td>
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<tr>
<td>DEAE Sephadex A-50 chromatography</td>
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<td>0.48</td>
<td>24.0</td>
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<td>Sephacryl S-200HR chromatography</td>
<td>5.2</td>
<td>1.42</td>
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<td>Protein-Pak DEAE 5PW chromatography</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Laccase I</td>
<td>1.8</td>
<td>3.00</td>
<td>10.8</td>
<td>42.9</td>
</tr>
<tr>
<td>Laccase II</td>
<td>0.3</td>
<td>1.67</td>
<td>1.0</td>
<td>23.9</td>
</tr>
</tbody>
</table>
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 (Vångvärd, 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 $g_0 = 2.197$ and $A_0 = 0.009$ cm$^{-1}$, and those of the type 3 Cu signal $g_0 = 2.263$ and $A_1 = 0.0176$ cm$^{-1}$. These values closely resemble those of Polyporus versicolor (2.190 and 0.009 cm$^{-1}$, and 2.243 and 0.0194 cm$^{-1}$, respectively; Vångvärd, 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. ostreatus 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 0.0090 cm$^{-1}$, and 2.243 and 0.0194 cm$^{-1}$, respectively; Vångvärd, 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. ostreatus laccase I is a copper-containing protein like other fungal laccases.
Table 2. Substrate specificity of laccase I from P. ostreatus

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

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 \( \mu \)M and \( 4.10 \times 10^9 \) nmol \( \text{O}_2 \) uptake min\(^{-1} \) (nmol enzyme\(^{-1} \)) and for syringic acid were 89 \( \mu \)M and \( 2.54 \times 10^8 \) nmol \( \text{O}_2 \) uptake min\(^{-1} \) (nmol enzyme\(^{-1} \)).

Single electron transfer

The EPR spectrum presented in Fig. 7 shows that 3,5-dimethoxy-4-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: \( a_{\text{CH}_3} = 14.36 \) mT (6 eq. protons), \( a_{\text{H(meta)}} = 14.36 \) mT (6 eq. protons) and \( a_{\text{H(ortho)}} = 3.40 \) mT (6 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-4-hydroxyacetophenone may be formed via deprotonation of the \( p \)-hydroxy group attached to the benzene ring.

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REFERENCES


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