Purification and partial characterization of laccase from the soft-rot fungus *Graphium* sp. M-1-9

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Synopsis

An extracellular laccase was isolated, purified, and partially characterized from the culture medium of *Graphium* sp. M-1-9, which causes soft rot in Japanese beech wood. Laccase produced by this fungus in cultures supplemented with copper sulfate was purified 60-fold by ammonium-sulfate precipitation, Sephadex G-100 gel-filtration chromatography, DEAE Sepharose ion-exchange chromatography, and Superdex G-75 gel-filtration chromatography, with a yield of 3.3%. Native and SDS-PAGE showed an active band with a molecular mass of approximately 67,000. Purified laccase maintained 70–80% stability at pH 6–10 for 180 min and at 30–40°C for 120 min. The optimum pH for the oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was pH 3, while that for the oxidation of 2,6-dimethoxyphenol (DMP), guaiacol, and syringaldazine was pH 6. Kinetic constants (K_m and k_{cat}/K_m) showed that 2,6-DMP and syringaldazine were more effective substrates than ABTS and guaiacol. Enzyme activity was completely inhibited by sodium azide or L-cysteine at a concentration of 1 mM. Laccase activity was also inhibited by 5 mM SDS.

Keywords: soft-rot fungus, laccase, *Graphium* sp., wood degradation

1. Introduction

Soft rot is caused by species of ascomycetous fungi, including asexual forms[1-3]. Typical soft-rot decay includes cavity formation in the S_2 layer of wood cell walls, erosion of the luminal surfaces of cell walls, and penetration by fine hyphae through cell walls [4]. During soft-rot decay, cellulose and hemicellulose are depleted at similar rates, whereas lignin is depleted at a slower rate [3, 5]. However, the mechanisms underlying wood degradation by soft-rot fungi, particularly lignin degradation, have not yet been examined in detail.

Several fungi have been shown to produce a complete system of extracellular cellulases and hydrolyze highly crystalline cellulose substrates[6]. Although a complete cellulase system alone appears to be insufficient for

degrading cellulose in wood cell walls, phenol oxidase decreases the lignin barrier, thereby allowing cellulases to access their substrates. The ratio of lignin loss to total wood mass loss, although always less than 1, is higher for soft rot caused by ascomycetes and deuteromycetes with significant phenol oxidase activity than by those with weak or no phenol oxidase activity [5, 7], suggesting that wood induces the production of phenol oxidase by soft-rot fungi. In addition, white-rot fungi, which preferentially degrade lignin over cellulose in wood, only do so in cultures with strong phenol oxidase activity [6, 8].

The isolation of *Graphium* sp. was previously reported by Kumagai and Tsunoda [9] from imported North American timber, such as Douglas fir (*Pseudotsuga menziesii* Franco) and western hemlock (*Tsuga heterophylla* Sarg.), that had been treated to prevent sap stain. Sap-stain treatments are economically very important because Douglas fir and western hemlock constitute Japan's major wood supply. Sap-stain fungi cause discoloration and disfiguration of wood in storage and in service. The development of stain in timber and the emergence of fungi on its surface are considered of great economic importance to the timber and wood preserving industries because of losses in commercial quality of the products. The dark pigments produced by fungi consume more bleach when stained wood is pulped, thereby increasing paper production costs. *Graphium* sp. is the asexual form of the ascomycete *Ceratocystis* sp., a well-known cause of sap stain [10]. *Graphium* sp. is categorized as a soft-rot fungus [3, 5] and also causes significant mass loss in hardwoods. We previously showed that *Graphium* sp. M-1-9 caused more than a 30% mass loss in Japanese beech wood in 2 months and more than a 50% loss in 4 months [5]. We also demonstrated that one-electron oxidation activity and phenol oxidase activity are involved in wood degradation by *Graphium* sp. M-1-9 [11, 12].

Laccase is a multi-copper oxidase that catalyzes the oxidation of an array of aromatic substrates concomitant with the reduction of molecular oxygen to water. It is one of the extracellular glycosylated enzymes primarily produced by white-rot fungi, but also by other fungi. Laccase plays an important role in the terrestrial carbon cycle by contributing to the degradation of lignocellulosic material such as wood [13]. Laccases have been purified and characterized from some asexual forms of ascomycetous fungi [14-18]. However, the relationship between laccases and wood degradation, particularly soft-rot wood degradation, has not been examined in detail [19]. The wood-colonizing ascomycete *Xylaria polymorpha* causes white rot [5, 20], and Liers et al. [21] reported that it produces laccase that may be involved in the oxidation of lignin. The findings of physiological studies and gene-specific sequencing indicate that laccase is the only ligninolytic oxidoreductase secreted by *X. polymorpha* and related fungi [21, 22].

We previously demonstrated that Japanese beech wood stimulated one-electron oxidation activity in four deuteromycete soft-rot fungi

including *Graphium* sp., as assayed by ethylene production from 2-keto-4-thiomethylbutyric acid and phenol oxidase activity, and that the one-electron oxidation activities of these fungi were linked to the rate of wood degradation. We also previously reported that laccase is a major source of phenol oxidase activity in soft-rot fungi. Although phenol oxidase activity is present when fungi are actively degrading wood, there is no relationship between phenol oxidase activity and one-electron oxidation activity or the rate of wood degradation, either over time or in total [11, 12].

In order to elucidate the mechanisms underlying wood degradation by the soft-rot fungus *Graphium* sp. M-1-9 in more detail, we isolated, purified, and partially characterized laccase from this fungus. This helps the protection of sap-stain and the environmental and industrial applications of laccase.

2. Materials and methods

2.1 Organism and culture conditions

Graphium sp. M-1-9 was isolated from untreated sapwood stakes (2 x 2 x 20 cm) of Japanese beech (*Fagus crenata* Blume) and Japanese cedar (*Cryptomeria japonica* D. Don) located under the floor of an occupied house [23, 24]. Stock cultures of the fungus were maintained on slants of potato sucrose agar.

Duncan's medium contained KH_2PO_4 (5.0 g), $MgSO_4 \cdot 7H_2O$ (4.0 g), K_2HPO_4 (4.0 g), NH_4NO_3 (6.0 g), and glucose (0.25%) per l of distilled water. $CuSO_4 \cdot 5H_2O$ (33 mg) was added to promote laccase production. pH was adjusted to 5.4~5.5. Wood particles (approximately 5 mm^3, 2.4 g) of Japanese beech were sterilized and sprinkled evenly over the surface of 30 ml of Duncan's medium with 0.5 g agar in 300-ml Erlenmeyer flasks. Wood particles were extracted twice with acetone while refluxing and dried before use. Cultures were inoculated with a small piece of the fungal mat and incubated at 28°C.

2.2 Enzyme assays

Laccase activities in culture filtrates and during purification were assayed spectrophotometrically using 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; ε_{420} = 3.6 x 10^4 M^{-1} cm^{-1}; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) [16] as a substrate. The reaction mixtures, containing 20 μl of the sample and 20 μl of 20 mM ABTS in 150 μl of 50 mM acetate buffer (pH 4.5) in microplates, were incubated at 37°C for 3 min. A_{415} was measured with a microplate reader (Bio-Rad Laboratories, Richmond, CA, USA). One unit of activity was defined as the amount of enzyme oxidizing 1 μmol of substrate min^{-1} ml^{-1}.

2.3 Enzyme purification

After a 3-week incubation, 50 mM acetate buffer (pH 4.5) was added to the culture. The mixture was stirred with a glass rod, shaken, and centrifuged at 10,000 x *g* at 4°C for 20 min. The filtrate was collected through filter paper, and ammonium sulfate was added to 50% saturation. The precipitate was removed by

centrifugation, and ammonium sulfate was added to the supernatant to 80% saturation. The resulting pellet was collected by centrifugation, dissolved in 20 ml of 50 mM acetate buffer (pH 4.5), and kept at -80°C until used.

Crude laccase was purified by gel filtration using a Sephadex G-100 column (3.8 x 70 cm) eluted with 50 mM acetate buffer (pH 4.5). The fraction with laccase activity was further purified with an ÄKTApurifier 10 system using a HiTrap DEAE Sepharose FF column (GE Healthcare Life Sciences, Uppsala, Sweden) equilibrated with 50 mM acetate buffer (pH 4.5), and the enzyme was eluted with 0–0.1 M NaCl in the above buffer at a flow rate of 0.5 ml min^{-1}. Laccase-positive fractions were pooled and applied to Superdex G-75 using the ÄKTApurifier 10 system equilibrated with 50 mM acetate buffer (pH 4.5). At each step, a Vivaspin 2-ml concentrator (MWCO: 10,000) (Sartorius**,** Goettingen, Germany**)** was used for desalting and concentrating.

The purified laccase preparation after Superdex G-75 was analyzed by anion-exchange chromatography in a Mono-Q 5/50 GL column (GE Healthcare) using the ÄKTApurifier 10 system to confirm purification. The column was equilibrated with 50 mM acetate buffer (pH 4.5), and proteins were eluted with a linear gradient of NaCl (0–0.3 M). Purified laccase was stored at -80°C and used for subsequent characterization.

2.4 SDS-PAGE and zymogram analysis

Samples after Superdex G-75 were denatured by boiling at 100°C for 5 min in sample buffer (2% SDS, 6% 2-mercaptoethanol, 10% glycerol, and 50 mM Tris-HCl buffer pH 6.8) and applied to an SDS-PAGE gel (5.0% stacking, 7.5% resolving). Following electrophoresis, the gel was silver-stained (Bio-Rad) or stained with Coomassie brilliant blue R-250 (Bio-Rad).

Non-denaturing PAGE was performed under similar conditions in the absence of SDS and 2-mercaptoethanol without boiling. The apparatus was kept at 4°C to retain enzyme activity. Laccase activity was visualized by staining with 20 mM ABTS in 50 mM acetate buffer (pH 4.5). The samples after HiTrap DEAE Sepharose FF were used for non-denaturing PAGE because the amount was very low after the final purification.

2.5 Effects of pH and temperature on *Graphium* sp. laccase stability and activity

The pH stability of the purified enzyme was investigated by measuring residual enzyme activity following an incubation in buffers of pH 2.0–12.0 for 30–180 min. Citric-phosphate buffer (50 mM) was used for pH 2.0–3.0 and pH 9.0–12.0, 50 mM acetate buffer was used for pH 3.0–5.0, and 50 mM phosphate buffer for pH 6.0–8.0. Reaction mixtures containing 185 µl of 50 mM acetate buffer (pH 4.5), 5 µl of enzyme solution, and 10 µl of 20 mM ABTS were incubated at 30°C for 1 min.

Thermal stability was examined by

measuring activity remaining after the enzyme incubation at temperatures between 30°C and 60°C for 120 min. Reaction mixtures containing 185 μl of 50 mM acetate buffer (pH 4.5), 5 μl of enzyme solution, and 10 μl of 20 mM ABTS were incubated at room temperature for 3 min.

The optimum pH for laccase activity with ABTS, guaiacol (Wako Pure Chemicals Industries, Ltd., Tokyo, Japan), 2,6-dimethoxyphenol (DMP) (Wako Pure Chemicals Industries, Ltd.), and syringaldazine (Sigma-Aldrich, St. Louis, MO, USA) as substrates (20 mM each) was estimated. Assays were performed at room temperature in the greater than pH 2–12 buffers. Reaction mixtures containing 185 μl of buffer, 5 μl of enzyme solution, and 10 μl of substrate were incubated at room temperature for 3 min.

The effects of temperature on laccase activity were measured with each of the above substrates at pH 4.5 at temperatures between 20°C and 70°C. Reaction mixtures containing 185 μl of 50 mM acetate buffer (pH 4.5), 5 μl of enzyme solution, and 10 μl of substrate were incubated at room temperature for 3 min.

The following wavelengths were used to measure optical densities with a UV-VIS spectrophotometer (SHIMADZU UV-1800, Kyoto, Japan): ABTS, 420 nm (ε_{420} = 3.6 x 10^4 M^{-1} cm^{-1}); 2,6-DMP, 470 nm (ε_{470} = 4.96 x 10^4 M^{-1} cm^{-1}) [25]; guaiacol, 436 nm (ε_{436} = 6.4 x 10^3 M^{-1} cm^{-1}); and syringaldazine, 530 nm (ε_{530} = 6.4 x 10^4 M^{-1} cm^{-1}) [16].

2.6 Substrate specificities of *Graphium* sp. laccase

Spectrophotometric measurements of substrate oxidation by purified laccase were performed at room temperature in a reaction volume of 200 μl. The enzyme solution was prepared as 0.2 U ml^{-1} after the Superdex G-75 gel-filtration step, and 20 μl was used in all assays. Substrate concentrations ranged between 5.0 x 10^{-5} M and 5.0 x 10^{-2} M. Measurements with ABTS were performed in 50 mM acetate buffer (pH 4.5). Measurements with 2,6-DMP, guaiacol, and syringaldazine were performed in 50 mM phosphate buffer (pH 6.0). Experimental data were analyzed with Lineweaver-Burk plots.

2.7 Inhibition of *Graphium* sp. laccase activity

The inhibitory effects of NaN_3, EDTA, L-cysteine, and SDS on laccase activity were examined with ABTS. The reaction mixtures contained 10 μl of 0.2 U ml^{-1} purified enzyme solution, 10 μl of 20 mM ABTS, and 1–20 mM inhibitor in 180 μl of 50 mM acetate buffer (pH 4.5) in a microplate. Enzyme activity was measured after a 3-min incubation at room temperature.

3. Results

3.1 Purification

A preliminary study indicated the activation of *Graphium* sp. M-1-9 laccase production by $CuSO_4$; thus, fungal cultures grown in Duncan's medium containing 0.13 mM $CuSO_4 \cdot 5H_2O$ were used in these

experiments. Although 2,5-xylidine had been shown to stimulate laccase production in other fungi [26], it did not affect *Graphium* sp. M-1-9 laccase production (data not shown).

Laccase activity was measured by the oxidation of ABTS at various purification steps. Laccase activity in *Graphium* sp. M-1-9 culture filtrates remained stable throughout a 5-h incubation at pH 4.0–4.5; however, activity was very rapidly lost at pH 3.0. Furthermore, laccase activity in culture filtrates was more stable at 4°C over 7 days than at room temperature or 28°C (data not shown). Therefore, acetate buffer (pH 4.0–4.5) was used for purification, and all purification procedures and the preparation of enzyme solutions were performed at 4°C or on ice.

Crude samples with laccase activity were obtained by the 80% ammonium-sulfate precipitation of *Graphium* culture filtrates. After the three subsequent purification steps, laccase had been purified approx. 60-fold with an overall yield of 3.3% (Table 1). Two peaks with absorbance at 280 nm were obtained after gel-filtration chromatography (Superdex 75); however, only the first peak exhibited laccase activity. Mono-Q anion-exchange column chromatography of this sample yielded a single peak with absorbance at 280 nm and laccase activity (data not shown); therefore, the peak from Superdex 75 was used as the final purification. The molecular mass of laccase was estimated to be approx. 67,000 by SDS-PAGE, and native PAGE yielded a band after activity staining with ABTS (Fig. 1). The concentrated purified enzyme solution was yellowish-brown in color, and there was no clear peak or shoulder on UV-vis spectra (data not shown).

Table 1. Purification of an extracellular laccase produced by *Graphium* sp. M-1-9

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Extracellular filtrate	2000	144.7	176.6	0.8	100	1
Sephadex G-100	12	26.0	1.2	21.7	18.0	27
Hitrap DEAE	1.0	8.4	0.2	42	5.8	52
Superdex 75	0.5	4.8	0.1	48	3.3	60

3.2 Effects of pH and temperature on *Graphium* sp. laccase stability and activity

The stability of purified *Graphium* sp. laccase was examined at pH 2–12 with ABTS as the substrate (Fig. 2). At pH 2.0, activity after 60 min was significantly higher than that after 30 min and then gradually decreased. Activities at pH 6–10 remained nearly stable at 70–80% for 180 min. After 30 min at pH 12, laccase activity was very low.

Purified laccase was rapidly inactivated by an incubation at 60°C for 30 min; however, it was stable at 30–50°C for 30 min and at 30–

40°C for 120 min (Fig. 3).

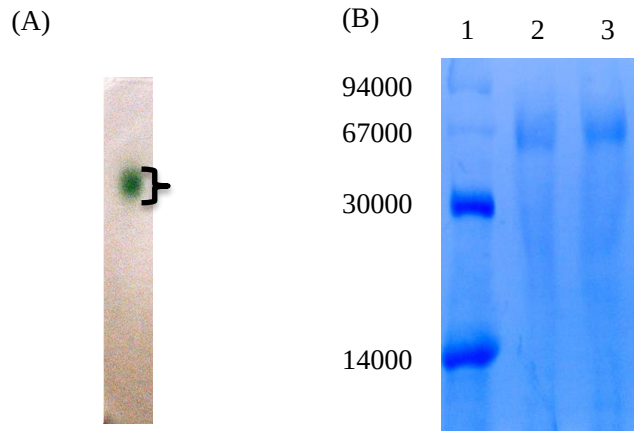

Fig. 1. Electrophoresis of purified laccase from *Graphium* sp. M-1-9.
(A) ABTS activity-stained native PAGE
(B) Denaturing SDS-PAGE of purified laccase (lanes 2 and 3), with molecular mass markers (lane 1).

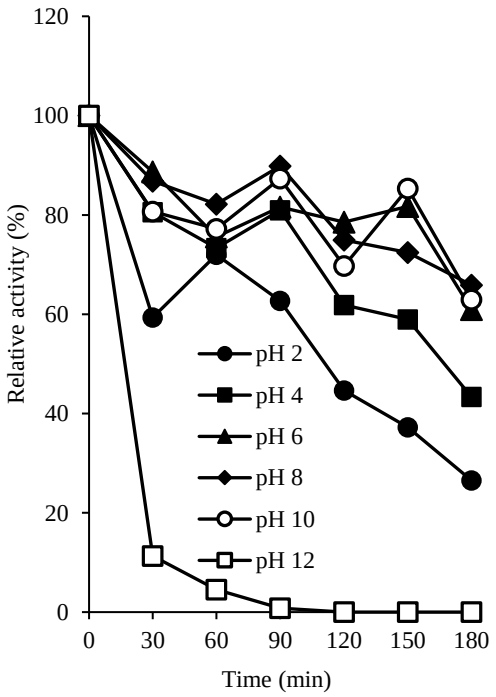

Fig. 2. pH stability of purified laccase from *Graphium* sp. M-1-9. Laccase activity was measured as described in Section 2.5, with 100% activity defined as the initial enzyme activity measured in a 1-min incubation with ABTS at pH 4.5 and 30°C.

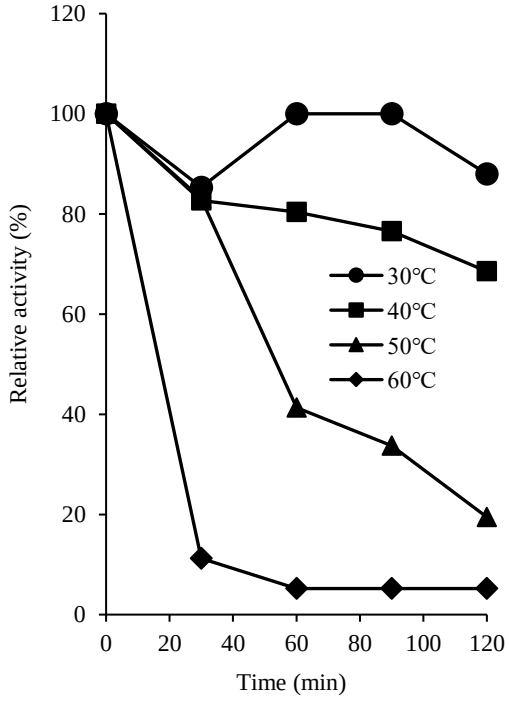

Fig. 3. Thermal stability of purified laccase from *Graphium* sp. M-1-9. Laccase activity was measured as described in Section 2.5, with 100% activity defined as the initial activity measured in a 3-min incubation with ABTS at room temperature and pH 4.5.

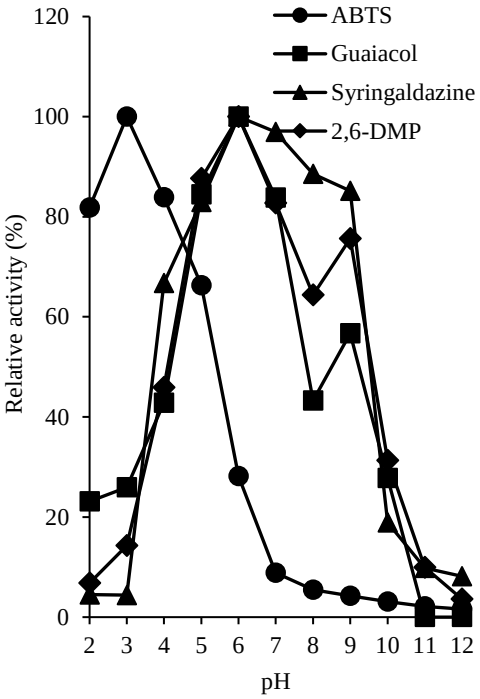

Fig. 4. Effects of pH on the activity of purified laccase from *Graphium* sp. M-1-9. Laccase activity was measured as described in Section 2.5. The highest activity was defined as 100% for each substrate.

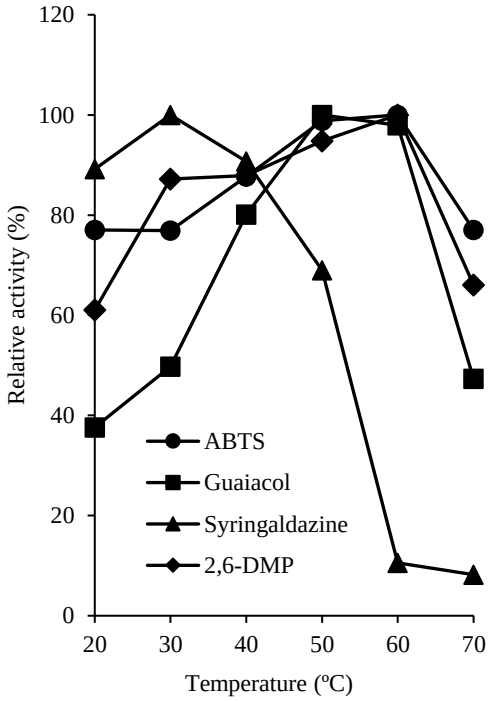

Fig. 5. Effects of temperature on the activity of purified laccase from *Graphium* sp. M-1-9. Laccase activity was measured as described in Section 2.5. The highest activity was defined as 100% for each substrate.

The activities of purified laccase on ABTS, 2,6-DMP, guaiacol, and syringaldazine were measured throughout the pH range (Fig. 4). Laccase activity with ABTS had a narrow range with an optimum at pH 3, a rapid decrease with increasing pH, and almost no activity at pH 8. The optimum for the three other substrates was pH 6. However, the oxidation of syringaldazine showed a broad optimum range between pH 5 and pH 9. The pH profiles for guaiacol and 2,6-DMP were similar between pH 5 and pH 9, with activity decreasing at pH 8 and increasing again at pH 9.

The activity of purified laccase was measured between 20°C and 70°C (Fig. 5). With ABTS and 2,6-DMP, the highest activity was at 60°C, whereas the highest activity with guaiacol was at 50°C and with syringaldazine at 30°C. Activity with syringaldazine markedly decreased at temperatures higher than 50°C.

3.3 Substrate specificities of *Graphium* sp. laccase

The specificities of laccase for several substrates are shown in Table 2. *Graphium* sp. laccase displayed the lowest K_m for 2,6-DMP, with the second lowest for syringaldazine and

the highest K_m for ABTS. The highest turnover number (k_{cat}) was with ABTS, followed by syringaldazine. The turnover number with ABTS was higher than that with guaiacol, whereas k_{cat}/K_m values were the same for both substrates. The k_{cat}/K_m value for 2,6-DMP was higher than those for the other three substrates.

3.4 Effects of inhibitors on *Graphium* sp. laccase activity

The sensitivity of purified laccase to several putative laccase inhibitors is shown in Table 3. Activity was completely inhibited by 1 mM NaN_3 and L-cysteine and by 5 mM SDS. EDTA reduced activity by 20% only at 20 mM.

Table 2. Substrate specificities of purified laccase from *Graphium* sp. M-1-9

Substrate	ε_{max} ($M^{-1}cm^{-1}$)	Wavelength (nm)	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m (μM^{-1} sec^{-1})
ABTS	36000	420	394	516	1.3
2,6-DMP	49600	468	13	228	17.1
Guaiacol	6400	436	113	147	1.3
Syringaldazine	65000	530	29	348	13.1

Table 3. Effects of inhibitors on the oxidation of ABTS by purified laccase from *Graphium* sp. M-1-9

Compound	Concentration (mM)	Inhibition (%)
NaN_3	1.0	100
L-Cysteine	1.0	100
SDS	1.0	0
	5.0	100
EDTA	1.0	0
	20.0	20

4. Discussion

4.1 Purification

Binz and Canevascini [27] reported that the purification of extracellular laccase from *Ophiostoma novo-ulmi*, an ascomycetous Dutch elm disease pathogen, was difficult because of fungal extracellular polysaccharides. Their yield of this laccase was 2.9%—very similar to the present results. The purification

of laccase from another ascomycete, *Myrioconium* sp. UHH 1-3-18-4, yielded 2%, although with 800-fold purification [17]. The molecular mass of purified *Graphium* laccase was consistent with those of most other fungal laccases, which have been reported to be between 60 kDa and 80 kDa [28].

Typical laccases show an absorption peak at approximately 600 nm, which is characteristic of type I Cu(II), and a shoulder at 330 nm, which indicates a type III binuclear Cu(II) pair [29-31]. Yellow laccases with the lack of characteristic absorption and atypical EPR spectra have been isolated from solid-state cultures of *Panus tigrinus* 8/18, *Phlebia radiata* 79, and *P. tremellosa* 77-51, although these enzymes from submerged cultures had a blue color [32]. Laccases from *Pleurotus ostreatus* D1 [33] and *Lentinus squarrosulus* MR13 [34] lacked the typical absorption spectrum of blue laccases. Thus, *Graphium* laccase appears to be similar.

4.2 pH and temperature

The effects of pH and temperature on *Graphium* laccase stability and activity are similar to those reported for *Fusarium proliferatum* laccase, in which the highest rate of ABTS oxidation was at pH 3–3.5, and maximum activity for ABTS was at 60°C. The stability of the two laccases at pH 6–10 was also similar, although *F. proliferatum* laccase was more stable at acidic pH and at 50°C [35].

The narrower pH range of *Graphium* laccase for the oxidation of ABTS than other substrates is similar to that reported for many other fungal laccases [36, 37]. Martin et al.[17] described a laccase from *Myrioconium* sp. UHH 1-13-18-4 with an optimum pH range of 2.5–3.0 for ABTS oxidation and no activity for ABTS detected above pH 7.5; furthermore, bell-shaped activity profiles showing distinct optima at pH 6.0 and pH 6.5 were observed for syringaldazine and 2,6-DMP oxidation, respectively. These pH optima are similar to those observed here with *Graphium* sp. M-1-9 laccase, although we observed a broader activity range for syringaldazine and 2,6-DMP. Xu [36] summarized that both the OH^- inhibition at the type II/type III center and the redox potential difference between a reducing substrate and the type I center could affect the pH activity profile of a laccase. The substrate such as ABTS is a nonphenolic compound and other three substrates are phenolic compounds, therefore the difference of pH profiles was obtained.

4.3 Substrate specificities

Kinetic constants have been measured for many fungal laccases using a number of methods and yielding widely differing findings [38]. Lower K_m values have generally been reported with syringaldazine and/or 2,6-DMP than with guaiacol or ABTS, similar to the results shown in Table 2. The laccase from the soil fungus in the family *Chaetomiaceae* had a higher K_m value with ABTS than with 2,6-DMP, guaiacol, and

syringaldazine [16]. Viswanath et al. [38] reported that k_{cat} values for a given laccase did not generally differ by more than 2–10 fold for different substrates, which is consistent with the results obtained with *Graphium* sp. M-1-9 laccase. As with *Graphium* laccase, purified laccases from the deuteromycete *F. solani* MAS2 and the *Chaetomiaceae* fungus showed the highest k_{cat}/K_m values with 2,6-DMP [16, 39]. Differences in k_{cat} values indicated that 2,6-DMP and ABTS were more effective substrates for *F. solani* MAS2 laccase than catechol or guaiacol [39], similar to the results reported here.

4.4 Inhibitors

Graphium laccase was 100% inhibited by 1 mM NaN_3 (Table 3). Laccases from other ascomycetes have been reported to be more tolerant of NaN_3, with *Cladosporium cladosporioides* laccase being inhibited by 5 mM NaN_3 [18], laccases from *Paraconiothyrium variabile* [40] and from a soil fungus of the *Chaetomiaceae* family [16] being inhibited by 10 mM NaN_3, and *Trichoderma harzianum* WL1 laccase being inhibited by 20 mM NaN_3 [29]. In contrast, purified laccases from the white-rot basidiomycetes *Pycnoporus cinnabarinus* [41] and *Coriolus hirstus* [42] were strongly inhibited by 0.1 mM NaN_3. Although 10 mM EDTA strongly inhibited *P. variabile* laccase [40], this concentration did not completely inhibit the activity of *Graphium* laccase or the other ascomycete laccases referenced above. The white-rot fungus *P. sanguineus* retained 92% activity at 25 mM EDTA [43]. *Graphium* laccase was completely inhibited by 5 mM SDS (Table 3). The complete inactivation of laccase from the deuteromycetous fungus *Myrothecium verrucaria* NF-05 by 5 mM SDS has been reported [44]. In contrast, 2 mM SDS increased the activity of *F. solani* MAS2 laccase to 130% [39].

4.5 Conclusions

The purified laccase from the soft-rot fungus *Graphium* sp. M-1-9 had a molecular mass of approx. 67,000 and oxidized ABTS, 2,6-DMP, guaiacol, and syringaldazine, which are typical substrates for laccase. Partial characterization demonstrated properties that were very similar to other fungal laccases. Therefore, this enzyme is involved in wood degradation by *Graphium* sp. M-1-9 in the same way as laccases derived from white-rot fungi. Our laboratory is currently optimizing media for increasing laccase production by this fungus for further characterization and biochemical applications, such as dye bleaching in the textile industry, bleaching of cork for bottled wine, and synthetic applications for phenols or peptides [45].

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軟腐朽を起こす *Graphium* sp. M-1-9 のラッカーゼの諸性質について

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要約

ブナ材に軟腐朽を起こす *Graphium* sp. M-1-9 を、硫酸銅を添加したブナ木片培地で培養し、ラッカーゼを分離・精製し、その諸性質を検討した。硫安沈殿、ゲルロ過クロマトグラフィー、イオン交換クロマトグラフィーにより 60 倍に精製されたラッカーゼの収量は 3.3%であった。SDS-PAGE により、分子量は約 67000 であった。精製したラッカーゼは pH 6-10 では 180 分間、また 30-40℃では 120 分間、70-80%の活性を維持した。最適 pH は基質により異なっており、2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)では pH 3、2,6-dimethoxyphenol (DMP)、guaiacol,、syringaldazine では pH 6 であった。K_m と k_{cat}/K_m より 2,6-DMP と syringaldazine は、ABTS と guaiacol より効率的な基質であった。酵素活性は 1 mM.のアジ化ナトリウムあるいは L-システイン、5 mM SDS で阻害された。