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Purification and Characterization of a Thermostable Laccase from *Trametes trogii* and Its Ability in Modification of Kraft Lignin

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A blue laccase was purified from a white rot fungus of *Trametes trogii*, which was a monomeric protein of 64 kDa as determined by SDS-PAGE. The enzyme acted optimally at a pH of 2.2 to 4.5 and a temperature of 70°C and showed high thermal stability, with a half-life of 1.6 h at 60°C. A broad range of substrates, including the non-phenolic azo dye methyl red, was oxidized by the laccase, and the laccase exhibited high affinity towards ABTS and syringaldazine. Moreover, the laccase was fairly metal-tolerant. A high-molecular-weight kraft lignin was effectively polymerized by the laccase, with a maximum of 6.4-fold increase in weight-average molecular weight, as demonstrated by gel permeation chromatography. Notable structural changes in the polymerized lignin were detected by Fourier transform infrared spectroscopy and ^{1}H NMR spectroscopy. This revealed an increase in condensed structures as well as carbonyl and aliphatic hydroxyl groups. Simultaneously, phenolic hydroxyl and methoxy groups decreased. These results suggested the potential use of the laccase in lignin modification.

Keywords: Laccase, white rot fungus, kraft lignin, polymerization, modification

Introduction

Laccase (E.C. 1.10.3.2) is a blue copper-containing oxidase found in plants, fungi, bacteria, and even insects [2, 19]. It catalyzes the oxidation of a broad range of substrates: monophenols, diphenols, polyphenols, methoxyphenols, aromatic amines, and some inorganic ions, and participates in the reduction of molecular oxygen to water [31]. Because of its high nonspecific oxidation capacity, laccase can be a useful biocatalyst for diverse biotechnological applications. These applications include paper pulp bleaching, textile dye decolorization, wine clarification, bioremediation, biosensors, and organic synthesis [25].

White rot fungi are the most common and efficient laccase producers. Laccase is ascribed a role in lignin degradation by white rot fungi, especially fungi without peroxidases [7]. It oxidizes phenolic moieties of lignin by one-electron abstraction. This yields phenoxy radicals that may undergo spontaneous fission reactions, which lead to the cleavage of carbon-carbon or carbon-oxygen bonds in lignin subunits [13]. In the presence of mediators, laccase is also able to oxidize non-phenolic lignin subunits [4]. However, *in vitro* oxidation with laccase usually causes polymerization rather than degradation of lignin, although some degradative reactions may occur simultaneously [9, 16, 18, 34]. Therefore, the specific role of laccase in lignin biodegradation remains unclear.

Although the *in vivo* function of laccase in lignin metabolism is not yet completely elucidated, recently the enzyme has attracted much attention because of its potential use in lignin modification. Lignin is one of the major constituents of the plant cell wall. It also comprises 15% to 30% of wood biomass and is the second most abundant biopolymer in nature, after cellulose. It is a three-dimensional amorphous polymer composed of three different phenylpropane units (p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) groups), which are linked *via* various ether and carbon-carbon bonds [32]. Currently, mostly as a byproduct of the kraft pulping process, huge amounts of industrial lignins are produced by the pulp and paper industry. Most of the kraft lignin (KL) from this process is directly burned in the pulp mill. This is done to

recover energy and chemicals and tends to be greatly under-utilized. Instead of being burned as low-value fuel, lignin has the potential to be used as an alternative raw material for producing more value-added products, such as adhesives, carbon fibers, resins, and plastics [5]. However, necessary modifications in lignin, such as an increase in molecular weight, have to be made to suite these applications. Enzymatic methods can be interesting in this case because of the advantages of enzymes, which include high specificity, mild reaction conditions, and utilization of benign oxidants. The oxidative polymerization activity of laccase makes it an attractive catalyst for lignin modification. Several studies have already been dedicated to examining the effects of laccase on the modification of KL [10, 15, 23, 30]. However, more studies are necessary to elucidate the detailed mechanisms of lignin modification by laccase.

Recently, we have isolated and identified a new strain of white rot fungus called *Trametes trogii* YDHSD. This strain produces a high level of laccase, which was found to exhibit high activity towards various industrial lignins in our previous studies [1]. The present work aimed to purify and characterize this laccase and to study its potential in lignin modification.

Materials and Methods

Organism and Culture Conditions

T. trogii YDHSD was isolated and identified by our laboratory (GenBank Accession No. EU790491.1). Stock cultures were maintained on wheat bran extract slant at 4°C and transferred every two months. Submerged cultivations were performed in 500 ml shaking flasks (120 rpm) containing 100 ml of wheat bran extract medium at 30°C. Four mycelial discs (7 to 8 mm in diameter) cut from 7-day-old plates were used to inoculate one flask.

Enzyme and Protein Assays

Laccase activity was determined spectrophotometrically using ABTS [2,2′-azino*bis* (3-ethylbenzthiazoline-6-sulfonic acid)] (ε_{420} = 36,000 $M^{-1}cm^{-1}$) as the substrate at 25°C. The standard assay mixture contained 1 mM ABTS, 20 mM sodium acetate buffer (pH 4.8), and a suitable amount of enzyme in a total volume of 3 ml. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute under the conditions described above. Protein concentration was determined by the Bradford method using bovine serum albumin as the standard.

Purification of Laccase

The supernatant from a 7-day submerged culture of *T. trogii* YDHSD was filtered and concentrated 20-fold using a stirred ultrafiltration system with a 10 kDa cutoff membrane (Amicon 8200; Millipore, USA). The concentrated supernatant was fractionated by ammonium sulfate precipitation. The 40−60% fraction was then dialyzed against 20 mM sodium acetate buffer (pH 4.8). The dialyzed sample was applied onto a DEAE-Sepharose FF column (1.6 × 12.5 cm GE Healthcare), which was equilibrated with the same buffer. Laccase was eluted with a linear 0 to 0.5 M NaCl gradient in 200 ml of the equilibration buffer at a rate of 1 ml/min. Active fractions were combined and concentrated. The concentrated sample was applied to a Sephacryl S-200 column (1.6 × 46 cm GE Healthcare) equilibrated with 50 mM phosphate buffer (pH 6.5) containing 0.15 M NaCl and eluted at 0.25 ml/min. Fractions with high laccase activity were pooled, concentrated, dialyzed against 50 mM phosphate buffer (pH 6.5), and stored at 4°C.

Electrophoresis and Spectral Properties

SDS-PAGE analysis of the purified laccase was performed using a 5% stacking gel and a 7.5% resolving gel. Protein bands were visualized by staining with Coomassie Brilliant blue R-250. The absorption spectrum of laccase was measured from 200 to 700 nm at 25°C in 20 mM sodium acetate buffer (pH 4.8) on a UV-3100 spectrophotometer (Shimadzu, Japan).

Effects of pH and Temperature on Laccase Activity and Stability

The effect of pH on laccase activity was examined with ABTS, 2,6-dimethoxyphenol (2,6-DMP), syringaldazine, and guaiacol as substrates over the pH range 2.2 to 7.0. This was in a 0.1 M citrate-phosphate buffer at 25°C. The stability of the laccase at various pH values was studied by incubating the enzyme at 25°C for 24 h and measuring the residual activity. The influence of temperature on the activity of laccase was determined over the temperature range of 25°C to 90°C with ABTS as the substrate in 0.1 M citrate-phosphate buffer (pH 4.8). To examine the thermal stability of laccase, the enzyme was incubated at different temperatures with the pH at 4.8, and the residual activity was measured periodically for 8 h.

Substrate Specificity and Kinetic Properties

The activity of laccase against various substrates was determined spectrophotometrically at 25°C and pH 4.0 in 0.1 M citrate-phosphate buffer. Kinetic constants for ABTS, DMP, syringaldazine, and guaiacol were determined by measuring initial velocities at various substrate concentrations at the optimal pH and 25°C.

Effects of Metal Ions and Inhibitors on Laccase Activity

The effects of metal ions and inhibitors on laccase activity were tested using ABTS as the substrate at pH 4.8 and 25°C in 20 mM acetate buffer. The purified enzyme was pre-incubated with the metal ion or inhibitor for 15 min; then, the remaining activity was measured.

Treatment of KL with Laccase

A commercial KL (Sigma-Aldrich) was treated with laccase at various pH values ranging from 3.0 to 8.0. Before treatment, the KL was dissolved in a minimum volume of 0.1 M NaOH. The KL

solution was then diluted to 1 mg/ml with 50 mM citrate-phosphate buffer and adjusted to the specified pH. Laccase was added to the mixture to obtain a concentration of 125 U/l, which initiated the reaction. The reaction was conducted in a 500 ml shaking flask (150 rpm) at 25°C. After 3 or 24 h, 200 µl of 5 M NaOH was added to 3 ml of the reaction mixture to terminate the reaction and dissolve the lignin. The obtained lignin solution was used for GPC analysis.

The KL treated at pH 6.5 was selected for structural analysis. After 24 h of treatment, the pH of the reaction mixture was adjusted to 2.4 with 6 M HCl to precipitate the lignin and terminate the reaction. The mixture was centrifuged at 10,000 ×*g* for 15 min. The precipitate was washed 10 times with acidified water (pH 2.4, adjusted with HCl) and then freeze-dried. It was then stored in a vacuum desiccator over phosphorus pentoxide. For control experiments, the same procedures were followed but with boiled laccase.

GPC Analysis of KL

Molecular weight distributions of KL were determined by GPC on a Sephacryl S-300 column (1.6 × 63 cm GE Healthcare) connected to a HPLC system (Shimadzu, Kyoto, Japan). The column was eluted at 1 ml/min with 50 mM aqueous NaOH solution. The effluent was monitored at 280 nm with a SPD-10 Avp UV-VIS detector. The column was calibrated with the following proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (250 kDa), alcohol dehydrogenase (141 kDa), bovine serum albumin (67 kDa), and horseradish peroxidase (40 kDa).

FTIR Analysis

FTIR was performed on a Nicolet NEXUS-470 FTIR spectrophotometer using the KBr pellet technique. Spectra were recorded in the range of 4,000 to 400 cm^{-1} with a resolution of 4 cm^{-1} and 64 scans. The spectra were baseline corrected and normalized to 1,510 cm^{-1} by Omnic software.

Elemental Analysis

The C, H, N, and S contents of the KL were determined on a Vario EL-III elemental analyzer. Oxygen content was calculated by the difference method.

^{1}H NMR Analysis

The KL was acetylated by dissolving 200 mg of sample in 6 ml of a 1:2 mixture of pyridine–acetic anhydrate in a 50 ml flask. The flask was purged with nitrogen for 2 min and left in the dark for 48 h at room temperature. The solution was then dropped into a 10-fold volume of acidified ice-cold water (pH 2.4). The precipitate was then filtered out, washed with acidified water, and dried under vacuum at 40°C. ^{1}H NMR spectra of 20 mg of the acetylated KL dissolved in 0.5 ml of DMSO-d6 were recorded on a Bruker Avance 400 spectrometer (400 MHz) using tetramethylsilane as the internal standard. For the quantification of protons, the signals in specified regions of the spectrum were integrated with respect to a spectrum-wide baseline drawn at the level of the background noise.

Results and Discussion

Purification and Molecular Properties of Laccase

Maximal laccase activity was achieved after 7 days of cultivation. Laccase was purified from the culture by ammonium sulfate fractionation and anion-exchange and gel filtration chromatography, as summarized in Table 1.

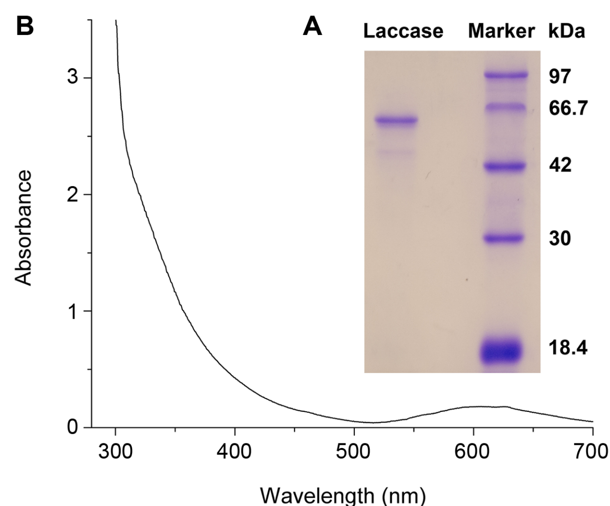

Fig. 1. Purification of laccase from strain YDHSD.
(**A**) SDS-PAGE of purified laccase from *T. trogii* YDHSD; (**B**) UV-visible spectrum of purified laccase from *T. trogii* YDHSD in 20 mM sodium acetate buffer (pH 4.8).

Table 1. Purification of laccase from *T. trogii* YDHSD.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Culture filtrate	4,032	210	19.2	1.00	100
Ultrafiltration	3,713	161	23.0	1.20	92.1
$(NH_4)_2SO_4$	3,161	81.5	38.8	2.02	78.4
DEAE-Sepharose FF	2,306	29.0	79.6	4.15	57.2
Sephacryl S-200	1,754	11.9	148	7.70	43.5

The enzyme was purified 7.7-fold to a specific activity of 148 U/mg protein with a yield of 43.5%. The homogeneity of the purified enzyme was confirmed by SDS-PAGE (Fig. 1A). The subunit molecular mass, based on SDS-PAGE, was 64 kDa. The molecular mass of the native enzyme was 54 kDa, as determined by gel filtration chromatography. These results indicated that the laccase is a monomeric protein. Most fungal laccases are monomeric proteins with a molecular mass between 60 and 70 kDa [3]. The UV-visible spectrum of the enzyme exhibited a shoulder at 320 nm (Fig. 1B). This is typical of a type III binuclear Cu^{2+} center. An absorption peak at 610 nm indicated the presence of a type I Cu^{2+} center, which is considered to be responsible for the blue color of the enzyme [27].

Effects of pH and Temperature on Laccase Activity and Stability

Like most other laccases, *T. trogii* laccase exhibited acidic pH optima, which were substrate-dependent. The optimum pH values for oxidation of ABTS, 2,6-DMP, guaiacol, and syringaldazine were 2.2, 3.0, 4.0, and 4.5, respectively (Fig. 2A). The enzyme was very stable at a pH of 6 to 9, retaining more than 86% of its initial activity after 24 h at this pH range (Fig. 2B).

The laccase showed a broad temperature optima ranging from 50°C to 75°C with maximum activity at 70°C (Fig. 2C). At room temperature (25°C), it displayed only 29% of the maximum activity. The enzyme was extremely stable below 50°C, with negligible activity loss after 24 h incubation at 40°C. Incubation at 50°C for 8 h only caused 10% loss of its initial activity (Fig. 2D). When incubated at 55°C for 8 h, it retained nearly 60% of its initial activity. The half-life of the enzyme at 60°C was about 1.6 h. It declined to 5 min at 70°C. The thermal stability of the enzyme was comparable to that of the thermostable fungal laccases reviewed by Hildén *et al*. [14]. This is a desirable property for industrial applications.

Substrate Specificity and Kinetic Properties of Purified Laccase

Like other laccases, the laccase from *T. trogii* YDHSD oxidized a broad range of phenolic substrates, such as 2,6-DMP, guaiacol, and syringaldazine. Non-phenolic substrates such as ABTS were also oxidized (Table 2). It is noteworthy

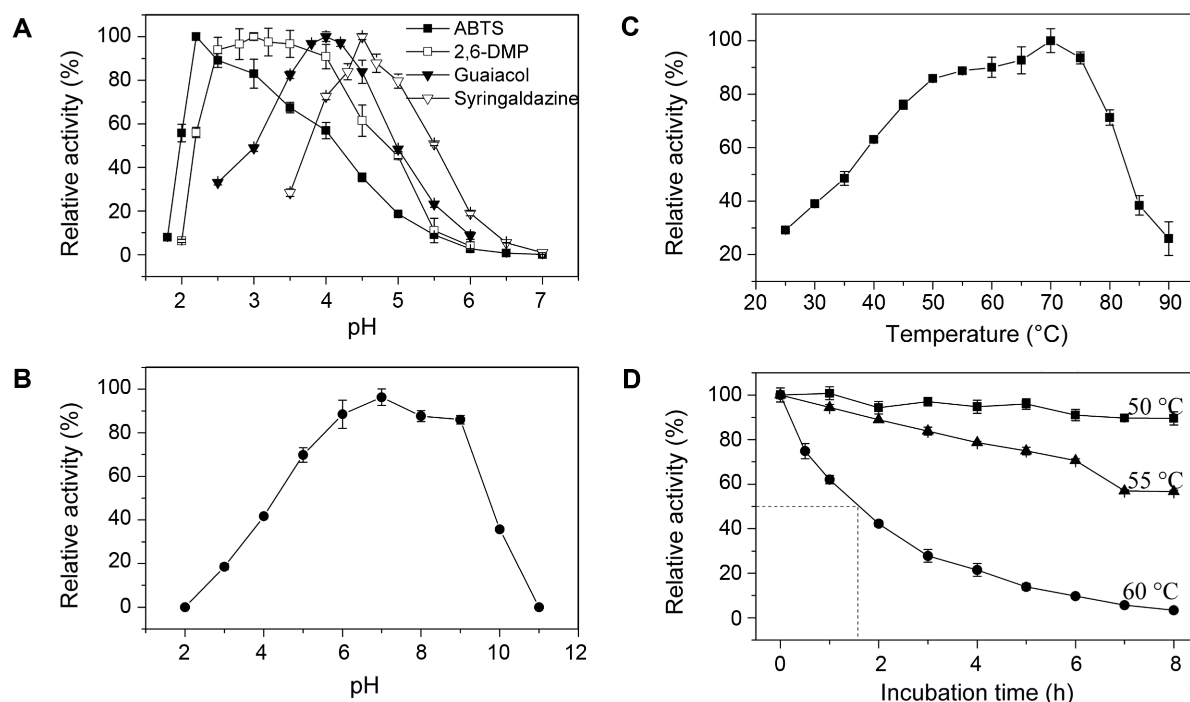

Fig. 2. Effects of pH and temperature on the activity and stability of the purified laccase.

(**A**) The effect of pH on the activity of laccase against different substrates; and (**B**) the effect of pH on the stability of laccase. The enzyme was incubated at 25°C for 24 h, and the residual activity was measured under the standard assay conditions. (**C**) The effect of temperature on the activity of laccase with ABTS as substrate at pH 4.8; and (**D**) the effect of temperature on the stability of laccase. The enzyme was incubated in 0.1 M citrate-phosphate buffer at pH 4.8. The residual activity was then measured under the standard assay conditions.

Table 2. Substrate specificity of laccase from *T. trogii* YDHSD.

Substrate	Concentration (mM)	λ (nm)	ε_{max}[a] ($M^{-1}cm^{-1}$)	Specific activity[b] (μmol/min/mg)
ABTS	1	420	36,000	142 ± 0.5
2,6-DMP	5	470	49,600	23.5 ± 3.3
Guaiacol	5	465	12,100	16.7 ± 0.5
Syringaldazine	0.01	525	65,000	15.8 ± 3.1
Catechol	5	450	2,200	13.2 ± 1.9
Pyrogallol	5	450	4,400	9.12 ± 0.67
Phloroglucinol	5	330	—	50.1 ± 3.6[c]
Ferulic acid	0.2	287	12,483	39.7 ± 1.0
Hydroquinone	1	248	17,542	36.9 ± 2.7
Methyl red	0.05	525	38,638	0.039 ± 0.001
Bromophenol blue	0.013	585	42,877	0.52 ± 0.01
Cresol red	0.1	435	16,928	2.96 ± 0.23
Phenol	2	270	—	0
Veratryl alcohol	5	280	—	0
Tyrosine	5	280	—	0

[a]Data for methyl red, bromophenol blue, and cresol red are from this study; others are from references.

[b]Results represent the mean ± standard deviation of triplicate determinations.

[c]Expressed as ΔOD/min/mg.

that the enzyme also oxidized the non-phenolic dye methyl red. There have been few reports of blue laccases that are able to directly oxidize methyl red without a mediator [21, 37]. Among the substrates tested, ABTS was the most readily oxidized, with a specific activity of 142 μmol/min/mg. As expected for a typical laccase, the enzyme showed no activity against tyrosine or veratryl alcohol.

The relationship between enzyme activity and substrate concentration was of the Michaelis-Menten type. The apparent kinetic constants were determined with four selected substrates that are commonly used for laccase assays (Table 3). As judged from the catalytic efficiencies (k_{cat}/K_m), ABTS was clearly the most preferred substrate, with a k_{cat}/K_m value of 35.6 $\mu M^{-1}s^{-1}$. Guaiacol was a relatively poor substrate, with the catalytic efficiency 1,000-fold lower.

Table 3. Kinetic constants of laccase from *T. trogii* YDHSD.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
ABTS	7.32	260	35.6
Syringaldazine	6.73	35.9	5.36
2,6-DMP	302	50.3	0.17
Guaiacol	552	21.1	0.038

The enzyme exhibited a K_m value of 7.32 μM for ABTS and 6.73 μM for syringaldazine, which were much lower than the corresponding values of most fungal laccases reported previously [3]. This indicated that the laccase from *T. trogii* YDHSD has exceptionally higher affinity towards these substrates.

Effects of Metal Ions and Inhibitors on Laccase Activity

Like laccases from *Pleurotus ferulae* and *Pycnoporus* sp. [6, 33], *T. trogii* laccase was strongly inhibited by Fe^{3+}, which caused 65.9% inhibition at the concentration of 1 mM and complete inhibition at 5 mM (Table 4). This may be due to the interaction of Fe^{3+} with the electron transportation system of laccase [29]. In contrast, other metal ions showed no significant inhibition at 1 mM concentration. The enzyme was even slightly activated by Cu^{2+}, Mn^{2+}, and Zn^{2+} at this concentration. The activation of laccase by certain metal ions, especially Cu^{2+}, has also been observed in other laccases, such as the laccase from *Cladosporium cladosporioides* [12]. The activation of laccase by Cu^{2+} may be due to the filling of type II copper binding sites with copper ions [21]. When the concentration increased to 10 mM, Al^{3+}, Ca^{2+}, K^+, Cu^{2+}, and Na^+ inhibited the enzyme to various extents, with the residual activity varied from 59.8% to 83.1%. In contrast,

Table 4. Effects of different metal ions on laccase activity.

Metal salt	Relative activity (% of control)		
	1 mM	5 mM	10 mM
KCl	100.1 ± 2.9	86.5 ± 1.3	78.5 ± 1.6
NaCl	96.8 ± 2.7	93.1 ± 1.3	83.1 ± 2.5
$CaCl_2$	96.6 ± 0.5	76.8 ± 1.8	68.8 ± 1.5
$CuSO_4$	102.3 ± 2.2	92.3 ± 0.7	80.9 ± 2.4
$MnSO_4$	102.7 ± 1.5	98.6 ± 2.7	91.8 ± 1.6
$ZnSO_4$	102.0 ± 2.5	99.3 ± 1.4	99.4 ± 4.3
$MgSO_4$	98.1 ± 1.1	95.1 ± 1.0	94.1 ± 1.8
$CoSO_4$	96.2 ± 0.9	89.5 ± 2.0	91.1 ± 1.3
$AlCl_3$	99.2 ± 1.5	89.0 ± 2.9	59.8 ± 2.0
$FeCl_3$	34.1 ± 2.7	0	0

Data represent the mean ± standard deviation of triplicate determinations. Laccase activity in the absence of metal ions was regarded as 100%.

Table 5. Effects of various inhibitors on laccase activity.

Inhibitor	Concentration (mM)	Relative activity (%)
EDTA	1	90.1 ± 4.0
	5	99.0 ± 4.8
	10	94.2 ± 1.4
SDS	1	101.4 ± 1.5
	5	54.2 ± 3.5
	10	41.4 ± 2.7
KI	1	100.9 ± 0.6
	5	74.5 ± 0.6
	10	71.1 ± 1.4
NaF	0.1	25.2 ± 4.9
	0.5	10.2 ± 1.2
	1	6.2 ± 1.1
NaN_3	0.1	0
DTT	0.1	0

Data represent the mean ± standard deviation of triplicate determinations. Laccase activity in the absence of inhibitors was regarded as 100%.

Mn^{2+}, Zn^{2+}, Mg^{2+}, and Co^{2+} only showed marginal inhibitory effect, while the relative activity remained above 91%. Compared with many other laccases [6, 17, 33, 36], the laccase from *T. trogii* was fairly metal-tolerant, supporting its potential for industrial applications such as bioremediation.

As for the tentative inhibitors tested, NaN_3 and DTT were the most effective. Both NaN_3 and DTT caused complete loss of laccase activity at 0.1 mM concentration, whereas EDTA was not an efficient inhibitor at all concentrations (Table 5). Similar results have been obtained for laccases from *Lentinula edodes* and *Echinodontium taxodii* [21, 28]. It has been reported that the binding of NaN_3 to the type II and III copper sites blocks internal electron transfer, thus inhibiting laccase activity [26]. DTT is a reducing agent of disulfide bonds. The strong inhibition of laccase activity by DTT indicated the importance of disulfide bonds for stabilizing the enzyme conformation. Most reported laccases were not strongly inhibited by EDTA, with only a few exceptions [6, 17, 35]. Interestingly, it seems that Cu^{2+} usually activated the laccases that were efficiently inhibited by EDTA. This may be explained by the relatively unstable binding of the type II copper to these laccases, which makes the copper easily removed by a chelator and the enzymes easily activated by exogenous Cu^{2+}. NaF was another effective inhibitor of the laccase, causing 75% inhibition at 0.1 mM concentration, whereas KI only showed mild inhibition. The inhibition of laccase by halogen anions was reported to be mainly competitive [24]. Compared with most other laccases, *T. trogii* laccase was not very sensitive to SDS, which showed no inhibitory effect at 1 mM concentration.

Polymerization of KL by Laccase at Different pH Values

Because pH is a critical factor influencing the laccase reaction, the activity of laccase towards KL was evaluated at different pH values. The weight-average (Mw) and number-average molecular weights (Mn) of the KL prior to treatment were determined to be 165,000 and 17,100, respectively. Both the Mw and Mn increased after laccase treatment at all pH values (Fig. 3). This indicated that polymerization of KL occurred, likely as a result of the radical coupling reactions initiated by laccase oxidation of the phenolic end groups in lignin. As a general trend, the polydispersity (Mw/Mn) of KL also increased, which was intimately related to the Mw increase.

As shown in Fig. 3, the degree of polymerization depended on both pH and treatment time. The pH optima for the polymerization were 6.0 for a treatment time of 3 h and 6.5 for a treatment time of 24 h. These pH optima were higher than those for oxidation of the low-molecular weight substrates such as ABTS and syringaldazine. Similar findings have been reported in other studies [10, 20]. Given the fact that laccase usually has acidic pH optima, whereas KL was more soluble at higher pH values, the pH profiles for KL polymerization may have been a result of a balance between enzyme activity and substrate availability. As seen in Fig. 3, at lower pH values (pH 3 to 6), the reaction was rapid and the majority of the polymerization was achieved already after the initial 3 h of reaction. However, the precipitation

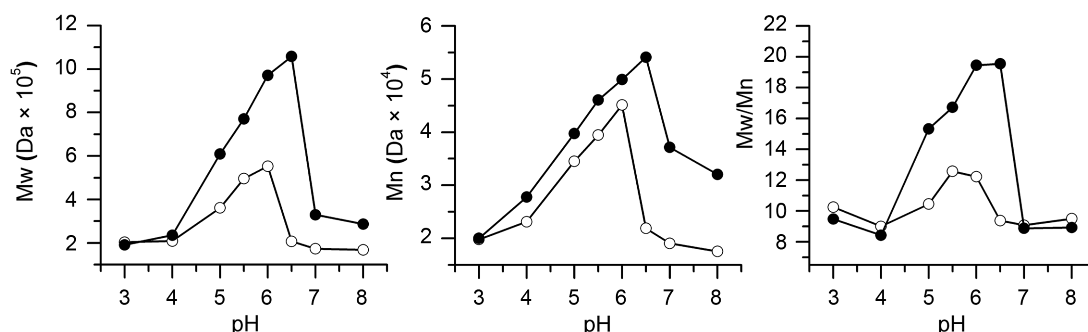

Fig. 3. Polymerization of kraft lignin by laccase at different pH values after treatment times of 3 h (open circles) and 24 h (solid circles).

of the polymer hindered its further polymerization. In contrast, although the polymerization was slower at higher pH values, the relatively higher solubility of lignin allowed it to reach a higher final molecular weight after a prolonged reaction time, such as the case at pH 6.5.

It has been reported that low-molecular-weight lignin is more reactive with laccase, whereas a high molecular weight is a handicap for lignin polymerization [22, 34]. Most of the lignins used for polymerization by laccase in previous reports had an initial Mw below 5,000 [9, 10, 23, 30]. In comparison, the KL used in this study had a relatively high initial molecular weight. Considering this, the polymerization of KL by the laccase was efficient, with a maximum of 6.4-fold increase in Mw after treatment for 24 h at pH 6.5.

FTIR Analysis

The structure of the KL treated by laccase for 24 h at pH 6.5 was analyzed by FTIR. The observed bands were assigned according to the literature [8]. As revealed by the FTIR spectra, the polymerized KL exhibited similar general structures to that of the untreated sample (Fig. 4). Nevertheless, changes in the relative intensities of certain bands were observed.

The most notable change was the increase in the band intensities at 1,716 and 1,652 cm^{-1}, which were assigned to unconjugated and conjugated C=O stretching, respectively. This suggests the enrichment of these structures in the polymerized lignin. Additionally, a strong increase in band intensity at 1,598 cm^{-1} and a slight increase at 1,268 cm^{-1} were observed. The band at 1,598 cm^{-1} was assigned to aromatic skeletal vibration plus C=O stretching. The band at 1,268 cm^{-1} was attributed to a G ring plus C=O stretching. However, it is believed that laccase alone does not modify the aromatic backbone of lignin. Therefore, the increase of these bands may have been due to C=O stretching rather than aromatic ring vibrations, considering that carbonyl groups have been reported to exert a significant influence on these bands [8]. The increase in carbonyl groups was most likely a result of Cα-oxidation, side chain cleavage, and quinone formation reactions. Similar results have been reported by Gouveia *et al.* [10].

Another structural change revealed by FTIR analysis was the formation of condensed structures in the treated lignin. As seen in the spectra, a weak shoulder appeared after treatment at around 1,325 cm^{-1}, which was attributed to G ring condensation (*i.e.*, G ring substitution at position 5). The formation of condensed structures was also demonstrated by the increase in the band intensity at 1,215 cm^{-1}, where contributions of C–C, C–O, and C=O stretching (G condensed > G etherified) were found.

A new band appeared at 1,371 cm^{-1}, corresponding to

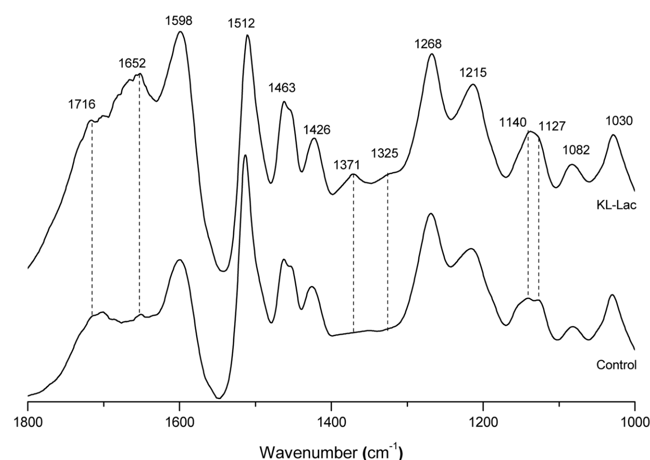

Fig. 4. FTIR spectra of kraft lignin before and after oxidation by laccase.

KL-Lac, kraft lignin treated by laccase.

aliphatic C–H stretching in CH_3 (not in OCH_3) and phenolic OH. This may have been caused by the introduction of methyl groups into lignin by enzymatic treatment.

For the untreated lignin, two overlapping bands could be distinguished at 1,100 to 1,170 cm^{-1}, corresponding to aromatic C–H in-plane deformation. The band centered at 1,140 cm^{-1} is typical for G units (G condensed > G etherified), whereas the band at 1,127 cm^{-1} is typical for S units. The intensity of the latter band decreased, and the former increased, after laccase treatment. It is a possibility that some syringyl moieties were demethoxylated and converted to guaiacyl moieties during treatment.

Elemental and ^{1}H NMR Analyses

According to the elemental analysis and the simple C9 formula calculated from Table 6, the polymerized KL contained a higher content of oxygen and a lower content of hydrogen. This is an indication that an oxidation reaction occurred.

The ^{1}H NMR spectra of the acetylated KL prior to and after treatment also revealed similar general structures (Fig. 5). However, differences in the contents of functional groups were still observed (Table 7). One of the most notable changes after treatment was the decrease in methoxyl protons, which decreased from 3.76/C_9 to 3.59/C_9. This suggests that demethylation occurred during oxidation by laccase. Another remarkable change was seen in the number of protons in aliphatic acetates, which increased from 2.23/C_9 to 2.41/C_9, indicating an increase in aliphatic hydroxyl groups. This was most likely due to the cleavage of side chains of lignin. Similar results have been reported by Sun *et al.* [30]. In contrast, the content of phenolic hydroxyl groups (PhOH) decreased to a slight extent, as indicated by the decrease in the number of protons in aromatic acetates, from 2.16/C_9 to 2.13/C_9. It appears that laccase oxidation of lignin does not necessarily lead to a significant decrease in PhOH, although they are the initial reactive sites for laccase. Niku-Paavola *et al.* [22] and Gronqvist *et al.* [11] each separately reported that the amount of PhOH in lignin remained constant during laccase treatment. One plausible explanation is that the initially formed phenoxy radicals are resonance-stabilized; therefore, the further reaction may occur at any of the reactive sites in the aromatic ring or side chain. Another explanation may be the generation of new PhOH *via* demethylation.

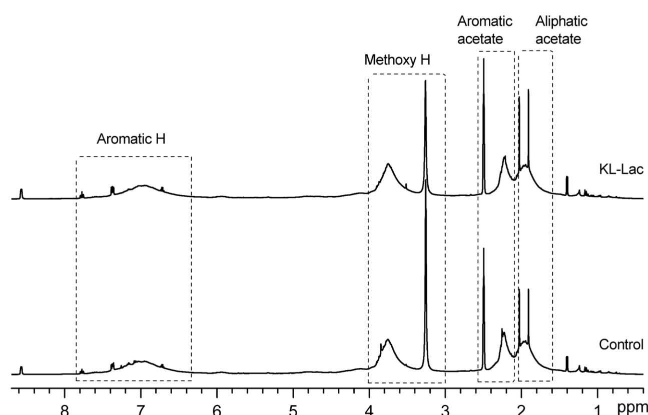

Fig. 5. ^{1}H NMR spectra of acetylated kraft lignin.

Table 6. Elemental analysis of kraft lignin.

Sample	Elemental analysis (%)					Simple C_9 formula
	C	H	O	N	S	
Control	64.26	5.92	28.46	0.25	1.11	$C_9H_{9.88}O_{2.99}$
KL-Lac	64.11	5.83	28.71	0.23	1.12	$C_9H_{9.75}O_{3.03}$

Table 7. Assignments of signals and protons per C_9 unit in the ^{1}H NMR spectra of acetylated lignins.

Signal (ppm)	Assignment	Protons/C_9	
		Control	KL-Lac
8.6	H in carboxyl and aldehyde groups	0.05	0.06
6.28–7.8	Aromatic H	2.22	2.18
3.00–4.02	Methoxyl H	3.76	3.59
2.10–2.52	H in aromatic acetates	2.16	2.13
1.60–2.10	H in aliphatic acetates	2.23	2.41
0.5–1.6	Aliphatic H	0.60	0.76

The amount of aromatic protons per C_9 unit decreased from 2.22 to 2.18 after treatment, indicating the condensation of the aromatic ring through C–C and C–O–C linkages. Additionally, the amount of protons in carboxyl and aldehyde groups increased from $0.05/C_9$ to $0.06/C_9$, indicating the enrichment of these groups. These results are consistent with the FTIR analysis.

In conclusion, a blue laccase was purified and characterized from the white rot fungus *T. trogii* YDHSD. The laccase exhibited high thermal stability and was moderately metal-tolerant. Moreover, the enzyme showed excellent catalytic activities, such as direct oxidation of methyl red and high affinity towards ABTS and syringaldazine. These features make the enzyme suitable for a wide scope of industrial applications. In addition, the laccase was able to catalyze the oxidative polymerization of a high-molecular-weight KL. The polymerization was pH-dependent. The favored pH range was 6 to 6.5, which was higher than that for the oxidation of low-molecular-weight substrates. A maximum of 6.4-fold increase in Mw was achieved at pH 6.5. Notable structural changes were detected in the polymerized KL. The carbonyl and aliphatic hydroxyl groups increased, whereas the methoxyl groups decreased. The phenolic hydroxyl groups also decreased, but to a lesser extent. Moreover, condensed structures were formed during the oxidation by laccase. These results indicate the potential use of the laccase in lignin modification and may provide useful references for revealing the mechanism of lignin oxidation by laccase.

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