# Purification and Characterization of a Laccase from *Cerrena unicolor* and Its Reactivity in Lignin Degradation

**Yousung Kim, Nam-Seok Cho,† Tae-Jin Eom,‡ and Woonsup Shin***

*Department of Chemistry, Sogang University, Seoul 121-742, Korea*
*†School of Forest Resources, Chungbuk National University, Cheongju 361-763, Korea*
*‡Department of Wood Science and Technology, Kyungpook National University, Daegu 702-701, Korea*
*Received February 20, 2002*

For efficient biopulping process, very active and stable lignase is essential. Laccase is one of the best enzyme in terms of environmentally benign processes, since the enzyme uses oxygen as an oxidant to degrade lignin and produces no harmful products. We could purify a laccase homogeneously from *Cerrena unicolor* in a very active state. It shows characteristic absorption feature with blue band at $\lambda_{max}$ = 604 nm. Molecular weight of the enzyme is 57,608 which could be accurately determined by MALDI/TOF MS. The enzyme has 2.8 copper ions per enzyme implying apoenzymes might exist together. The enzyme is active in lignin degradation and the activity increases 4 times in the presence of ABTS as a mediator.

**Keywords :** Laccase, *Cerrena unicolor*, Purification, Lignin, Degradation.

## Introduction

The pulp bleaching process is regarded as one of main causes in contaminating environments since pulp and paper industries have used a lot of chlorine to remove residual lignin. The formation and release of chlorinated organic compounds during this process is enormous and there is a big pressure to reduce the amount of chlorine in bleaching process.[1] The process is an oxidative degradation of lignin by chlorine. Oxygen, peroxide, ozone might be better oxidants for the environment although they are effective or more expensive than chlorine. In case of using oxygen and peroxide, environmentally benign and efficient catalysts are essential since the reactivity is less than that of chlorine.[2] Cellulases, hemicellulases including xylanases, manganese peroxidases, laccaeses are enzymes developed for pulp and paper processing.[3] Cellulases have used for pulp fibrillation to enhance or restore fiber strength properties and for improving the strength of paper by increasing fiber-fiber contact.[1] Xylanases have used to pre-bleach the lignin to reduce the amount of bleaching chemicals to obtain a given brightness.[1] It is suggested that xylanase depolymerizes xylan blocks and increases accessibility or helps liberation of residual lignin by releasing xylan-chromophore fragments.[5] Manganese peroxidase, lignin peroxidase and laccases are lignin-oxidizing enzymes and are mainly obtained from lignin-degrading (white-rot) fungi.[6] Peroxidases need hydrogen peroxide to oxidize lignin, while laccase uses oxygen as an oxidizing agent to degrade lignin. It is reported that manganese peroxidase can oxidize phenolic units of lignin[7] and lignin peroxidase can oxidize both phenolic and non-phenolic units.[8] Laccase is known to oxidize phenolic units of lignin and be able to oxidize non-phenolic units in the presence of mediators.[9]

Laccase (*p*-diphenol:dioxygen oxidoreductase, EC 1.10.3.2) is a multi-copper oxidase having type 1, type 2, and type 3 copper sites and can be obtained from both tree and fungi.[10] Laccase is currently being investigated by many researchers for biosensors[11] and biofuel cell applications,[12] and for industrial degradation processes. Laccases are known to catalyze both initial polymerization of monolignols to oligolignols and depolymerization/degradation of lignin.[6] Fungal laccases are active in degrading lignin and extensive efforts are being paid to search for highly reactive and stable laccases.[13] The enzyme is also useful in detoxification and decolorization since various kinds of aromatic compounds can be used as substrates.[14] Laccase couples catalytic oxidation of lignin with 4 electron reduction of oxygen. The final product from oxidant is a water without any harmful intermediate to make laccase the best candidate for the environmentally benign bleaching process. There have been a lot of efforts to purify stable and active laccase from many different sources such as *Rhus vernicifera, Rhus succedanea, Acer pseudoplatanus, Pinus taeda, Polyporus versicolor, Neurospora crassa, Pleurotus ostreatus, Polyporus pinsitus, Rhizoctonia solani, Myceliophthora thermophila, Scytalidium thermophilum, Polyporus anisoporus, Phlebia radata, Podospora anserina, Myrothecium verrucaria*, etc.[10,15] Also cloned laccases were appeared.[16,17] There are some reports for the purification of laccase from *Cerrena unicolor*, but the finally purified enzyme activity is not satisfactory.[18,19] We report here that an active laccase could be isolated and purified from *Cerrena unicolor* from the culture collection of the School of Forest Resources, Chungbuk National University, Cheongju, Korea and investigated the enzyme properties and reactivies.

## Experimental Section

**Reagents and instruments**. Chemicals used for buffers

*To whom correspondence should be addressed. Tel: +82-2-705-8451; Fax: +82-2-701-0967; E-mail: shinws@ccs.sogang.ac.kr

and substrates were at least reagent grade. ABTS was purchased from Aldrich and stored under inert atmosphere before use. Milli Q (Millipore) water purification system was used for preparing aqueous solutions. HP8452A or 8453 diode array spectrometer was used to obtain UV/VIS absorption spectra and kinetic measurements. The methanol concentration after lignin degradation was quantified by HP5890 Series II plus gas chromatography equipped with a packed stainless steel column (1/8" by 5 ft.) containing chromosorb 102 (John-Manville Co.) and flame ionization detector.[20] The injector and detector temperatures were 180, 150 °C, respectively. The oven temperature was programmed from 75 to 95 °C at a gradient of 5 °C/min initially and 95 to 200 °C at a gradient of 20 °C/min, then held at 200 °C for 5 min. Nitrogen was used as a carrier gas at a flow rate of 30 mL/min. Protein purity and approximate molecular weight were determined by SDS-PAGE (Bio-Rad Miniprotien II) using 12% polyacrylamide. To determine more accurate molecular weight of the protein, MALDI-MS (Matrix assisted laser desorption ionization mass spectrometer) with TOF (Time-of-flight) detector was used (Kratos Kompact MALDI II, England).

**Cultures**. The white rot fungus *Cerrena unicolor* (CFC-120) was from the culture collection of the School of Forest Resources, Chungbuk National University, Cheongju, Korea. The fungus was maintained on 2% (wt/vol) malt agar slants. Pieces of mycelium (*ca.* 0.5 cm) were transferred from the agar slant into the sterile (0.1 MP, 45 min.) liquid medium (70 mL in 250 mL conical flask containing glass beads). The medium composition is adapted from Wojtas-Wasilewska *et al.*[21] omitting Difco yeast extract. Before sterilization, pH of the medium was adjusted by 1 M hydrochloric acid to 5.6. The culture was grown at 26 °C under unstirred conditions. When the mycelium occupied the whole surface of the liquid the mycelinal mats were broken by shaking with the beads and the homogenate was transferred (10% of total volume of the medium) into 3 L of sterilized medium of the same composition as above. The submerged aerated culture (30 L of sterilized air per liter of the culture per hour) was grown for 12 days without temperature control. The grown culture was filtered through Miracloth (Calbiochem, Lucerne, Switzerland). The filtrate was concentrated to *ca.* one tenth of the volume at 4 °C with an Amicon ultrafiltration system equipped with a filter type PTGC (pore size 10,000 NMWL).

**Purification of laccase**. The solution was centrifuged at 9,000 rpm for 50 minutes and the supernatant was collected and extensive buffer exchange was performed using stirred ultrafiltration cell (Amicon 8200, YM-30 membrane) with 0.01 M acetate buffer (pH = 6.0). The solution was then applied to a DEAE sephacel (Pharmacia) column and laccase active fractions were pooled together after step elution from 0.05 to 0.5 M KCl by 0.05 M increment. The laccase active fractions were eluted at 0.3 M KCl. The pooled enzyme solution was dialyzed against 0.05 M acetate buffer (pH = 6.0) by the ultarfiltraton cell and applied to a CM sepharose (Pharmacia) column. The solution eluted from the column was all combined and applied to another DEAE sephacel column and eluted similarly except 0.025 M KCl step increment. The laccase active fractions are eluted at 0.15 M KCl and those were pooled and dialyzed against 0.01 M acetate buffer (pH = 5.7) and concentrated. Finally, the solution was applied to a sephacryl S-100 (Pharmacia) column and eluted with the same buffer. The laccase active fractions were pooled and conducted extensive buffer exchange with 0.1 M acetate buffer solution (pH = 5.7). The enzyme solution was concentrated with centrifugal filter units (Centricon, Amicon, YM-30 membrane) and stored at -4 °C refrigerator. Laccase activity was determined by oxidation of ABTS.[17] A suitable amount of enzyme was added to 1 mM ABTS solution in pH 5.3 of 8 mM MES buffer, and oxidation of ABTS was followed by absorbance increase at 405 nm ($\varepsilon_{405} = 35{,}000\ M^{-1}cm^{-1}$). Protein concentrations were determined by Biuret method using BSA as a standard[22] and copper ion content was spectrophotometrically determined by 2,2'-biquinoline.[23]

**Lignin degradation**. Hydrolytic lignin purchased from Aldrich was used for degradation experiment. 5 mL of 20 mg/mL of the lignin solution was prepared in 0.1 M acetate buffer (pH = 6.0) in 10 mL round flask and stirred under 1 atm oxygen. The reaction was initiated upon adding 5 units of laccase. If mediator is needed, ABTS was added to the solution to make 1 mM concentration before adding the enzyme. The sample for GC analysis was taken 90 $\mu$L in every 10 minutes with micro-syringe during the reaction and 10 $\mu$L of 50 mM sodium azide solution was added to stop the reaction. The sample was centrifuged to remove residual lignin and kept frozen in liquid nitrogen until GC measurements.

## Results and Discussion

**Enzyme purification**. Table 1 shows the results of purification of laccase from *Cerrena unicolor* at each purification step. The specific activity of the first filtered solution is 16

**Table 1**. Purification of laccase from *Cerrena unicolor*

| Purification step | Amount of protein (mg) | Specific activity (unit/mg) | Total activity (unit) | Turnover number ($min^{-1}$) | Recovery (%) |
|---|---|---|---|---|---|
| Filtered & ultrafiltrated | 500 | 16 | 8,000 | 930 | 100 |
| Centrifuged & buffer exchanged | 200 | 34 | 6,400 | 2,000 | 80 |
| DEAE sephacel I | 33 | 160 | 5,300 | 9,400 | 67 |
| CM sepharose – DEAE sephacel II | 17 | 150 | 2,600 | 8,400 | 33 |
| Sephacryl S-100 | 13 | 140 | 1,800 | 8,100 | 23 |

units/mg which is 320 times higher than previously reported one.[18] although the condition of determining activity is a little different (1 mM ABTS solution in pH 5.3, 8 mM MES buffer *vs.* 3 mM ABTS in pH = 3.5, 100 mM glycine buffer). Gianfreda et. al showed that the turnover is maximized at 3 mM ABTS and pH optimum for the activity measurement is about 3.5, while the optimum pH is 5.5 in our case.[24] The pH or substrate concentration could affect the activity of the enzyme. The laccase activity of *Cerrena unicolor* is known to vary by growing conditions season, temperature, etc.[25] We grew the fungus in spring, which is the best growing season, in the outside of laboratory without temperature control for 3 L medium.

Centrifugation and extensive buffer exchange against YM-30 membrane could remove more than half of the other proteins and specific activity increased about twice from 16 to 34. It is obvious that this procedure is very effective and can remove low molecular weight proteins and small molecules. About 20% loss in total activity occurred in this step.

The main increase in specific activity was obtained after running DEAE sephacel column. The specific activity increased 5 times (from 34 to 160) and more than 80% of other proteins were removed. The specific activity of 160 units/mg is one of the most active laccase ever reported.[13,17,18] The enzyme solution looks brownish blue color implying other brown colored proteins are still remained although the activity is quite high. The absorption spectrum of this solution showed a sharp absorption around 400 nm on the shoulder of blue band, which is a characteristic Soret band from heme containing proteins. We tried to remove the proteins by CM sepharose and 2-nd DEAE sephacel columns, but heme containing proteins are still shown in the absorption spectrum of the laccase solution. The total activity dropped down to half and the specific activity is about the same in this step, which means the process is not useful and only gave losses of the active enzyme. Sephacryl S-100 gel filtration column was effective to remove the remaining heme proteins from the laccase. The homogeneous protein at this stage was verified by the SDS-PAGE (Figure 1) showing single band without other contaminating bands. Laccase normally exists as an monomeric structure.[19] The absorption spectrum of the enzyme solution shows no heme band around 400 nm. There is a small feature around 330 nm, which is known as the ligand to copper charge transfer from type 3 site. The blue band of this laccase shows an absorption maximum at 604 nm with extinction coefficient of 3,540 $M^{-1}cm^{-1}$, which is characteristic for the blue copper proteins having absorption band around 600 nm with extinction coefficient in the range of 3,000 and 7,000 $M^{-1}cm^{-1}$.[26] The $A_{280}/A_{604}$ ratio is 19.3 which is also typical for normal laccases.

It should be noted that Bekker *et al.*[19] obtained the homogeneous laccase with 1.5-fold purification (33 to 50 units/mg) from the same organism. Gianfreda *et al.*[18] could purify 120-fold (0.05 to 6.0 units/mg), but both initial activity from the culture and the final activity of the purified

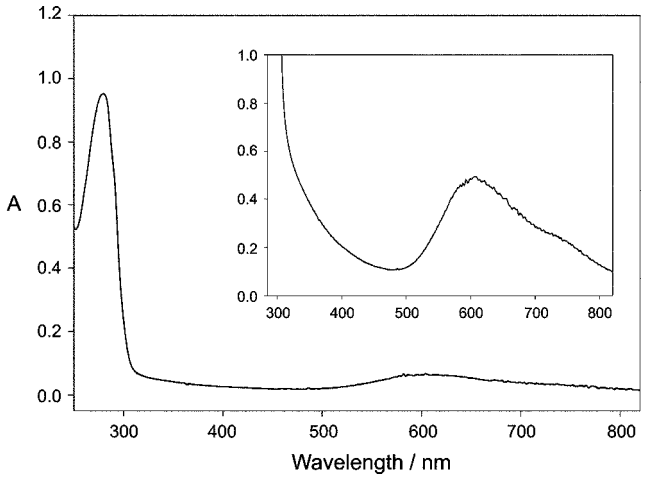


**Figure 1**. Absorption spectrum of laccase from *Cerrena unicolor* (0.90 mg/mL in pH 6.0, 0.1 M acetate buffer, at room temperature, 8.8 mg/mL for inset).

laccase is far below 1/10 of the present purification. The turnover number of the purified enzyme is 8,100 $min^{-1}$ which is also much higher than laccases from other sources, 2,700 $min^{-1}$ for *Polyporus pinsitus*, 2,400 $min^{-1}$ for *Rhizoctonia solani*, 440 $min^{-1}$ for *Myceliophthora thermophila*, 45 $min^{-1}$ for *Scytalidium thermophilum*, 430 $min^{-1}$ for *Myrothecium verrucaria*.[17]

Laccase active fractions were eluted at 0.3 M KCl at the 1-st DEAE column, while those were eluted at 0.15 M KCl at the 2-nd DEAE sephacel column. It seems small molecules and other proteins removed after 1-st DEAE sephacel column affect electrostatic properties of laccase itself or DEAE sephacel resin and change the elution behavior of the anion-exchange resin. It is known that laccases have a cooperative action in degrading lignin with other proteins or small organic mediators. The interactions among them are

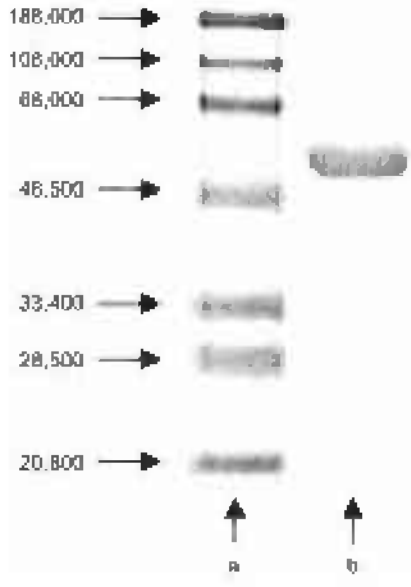


**Figure 2**. SDS polyacrylamide gel electrophoresis of laccase from *Cerrena unicolor*. (a) standard proteins, (b) finally purified enzyme.

actively being investigated.[27]

**M.W. determination**. Molecular weight of the laccase was determined roughly according to the electrophoretic mobility of protein in polyacrylamide gel in the presence of sodium dodecyl sulfate compared to the molecular weight standards. Since the electrophoretic mobility is a function of molecular shapes, the above method is not precise if three-dimensional structure of the molecule in solution is not known. The approximate molecular weight of the laccase purified in this study could be estimated to be 60,000 (Figure 1). Recently, mass spectrometric determination for the molecular weight of large molecules became common.[28] and we used MALDI/TOF MS system to determined the molecular weight of the purified laccase. The mass spectrum shows the strong parent peak at m/z = 57,608, which gives the best measurement for the molecular weight of the purified laccase (Figure 3). The peak at 29,005 is from doubly charged parent molecule. The molecular weights of laccases from various fungi lie in the range of 40-80 kDa[10] and the published molecular weight of laccase from *Cerrena unicolor* is 66 kDa which was measured by SDS-PAGE.[19]

**Copper content of the laccase**. Laccase is a muti-copper oxidase having 4 copper ions per enzyme as metal clusters called type 1 of monomeric blue copper site, type 2 of monomeric normal copper site, and type 3 of binuclear copper site. It is common to determine copper content for the newly purified laccase. The copper content of the purified laccase was determined spectrophotometrically at 546 nm based on the development of strong absorption by cuprous-biquinoline complex.[23] It is measured to be 2.8 copper ions per laccase which is smaller than the expected value of 4. The uncertainties in determining copper concentration and protein concentration could affect the results. The error range for each measurement is estimated to be 5-10%. Other possibility of being low number of copper content may be

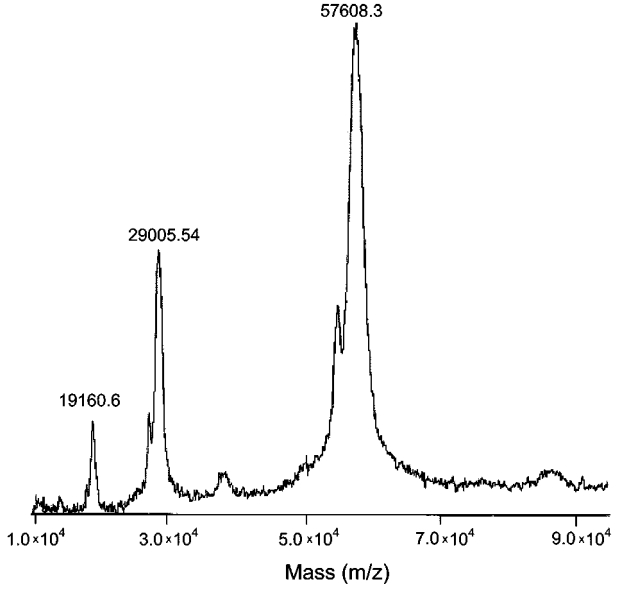


**Figure 3**. MALDI-TOF mass spectrum of laccase from *Cerrena unicolor*.

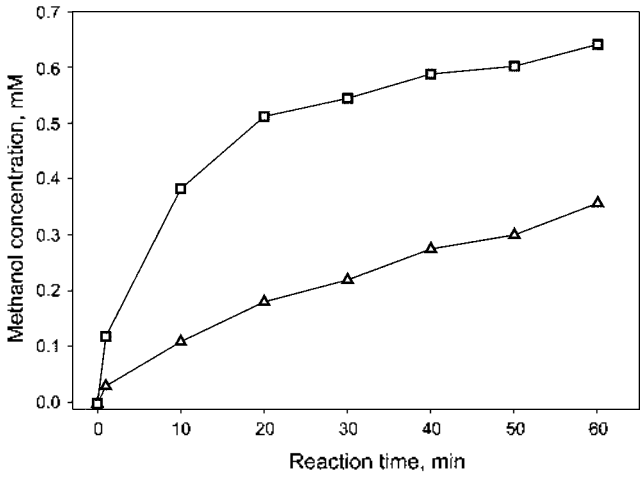


**Figure 4**. Time course of lignin degradation by laccase and ABTS. (a) lignin (20 mg/mL) + laccase (1 unit/mL), (b) lignin (20 mg/mL) + laccase (1 unit/mL) + ABTS (1 mM)

the existence of apoproteins which are missing copper ions in the active sites.

**Activity toward lignin degradation**. The activity of degrading lignin is measured by the increase in concentration of methanol, which is from the cleavage of methoxy group of lignin.[29] It is known that kappa number decrease, which is an index of bleaching, correlates well with the amount of methanol formation by lignin degradation.[14] Laccase by itself has a capability to destroy lignin under oxygen, however, the reaction rate increases significantly in the presence of mediators.[30] The laccase purified in the current study was applied to degrade lignin and results are shown in Figure 4. The enzyme is active in destroying lignin and the rate increases more than 4 times in initial 10 minutes upon adding 1 mM ABTS. Since ABTS is known to deactive laccase[31], the degrading reaction diminishes quickly after 20 minutes. Although ABTS is very active mediator, many researchers are actively looking for other mediators since ABTS deactivates laccase and is very expensive.[32] The amount of methanol formed after 1 hour is 0.65 mM, which can be converted to 21 mg/L of methanol. The number is equivalent to the kappar number decrease of 5.0 which comparable to the published results.[33]

## Conclusions

We could purify a highly active laccase homogeneously from *Cerrena unicolor*. The absorption spectrum, molecular weight, copper content, and lignin degradation activity are well matched with characteristic features of laccases.

**Acknowledgment**. This work was supported by Korean Research Foundation (KRF 2000-042-G00016). Thanks to Korea Basic Science Institute to obtain MALDI-TOF MS data.

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