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Cho, Nam-Seok

Wood and Paper Science, Chungbuk National University

Leonowicz, Andrzej

Department of Biochemistry, Maria Curie-sklodowska Univeristy

Jarosz-Wilkolazka, Anna

Department of Biochemistry, Maria Curie-sklodowska Univeristy

Ginalsika, Grazyna

Department of Biochemistry, Maria Curie-sklodowska Univeristy

他

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## Degradation of a Non-Phenolic $\beta$ -0-4 Lignin Model Dimer by *Cerrena unicolor* Laccase and Mediators, Acetovanillone and Acetosyringone

Nam-Seok CHO<sup>1</sup>, Andrzej LEONOWICZ<sup>2</sup>, Anna JAROSZ-WILKOLAZKA<sup>3</sup>,  
Grazyna GINALSKA<sup>3</sup>, Hee-Yeon CHO<sup>3</sup>, Soo-Jeong SHIN<sup>1</sup>,  
Yun-Jeong CHOI<sup>1</sup> and Shoji OHGA\*

Laboratory of Forest Resources Management, Division of Forest Ecosystem Management,  
Department of Forest and Forest Products Sciences, Kyushu University,  
Sasaguri, Fukuoka 811-2415, Japan

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This study focuses on the laccase and mediator co-operation in modelling lignin dimer veratrylglycerol- $\beta$ -vanillate ether (VVE) oxidation. Earlier we stated that constitutive laccase isolated from *Cerrena unicolor* can cooperate with acetovanillone (AV) and acetosyringone (AS) in degradation of high molecular fraction of lignosulfonates. The fungus *C. unicolor* is known as a very good producer of cheap constitutive laccase. Its cultivation for this reason does not require stimulation by the expensive and toxic inducer like 2,5-xylidine. The main purpose of present study is to examine capability of *C. unicolor* laccase to oxidize VVE, which is a non-phenolic lignin model compound with external mediator HBT, and AV and AS in order to choose one, most efficient system. In the presence of laccase, generation of monomeric aromatic acids from non-phenolic lignin model dimer veratrylglycerol- $\beta$ -vanillate ether (VVE) was observed. The addition of acetovanillone (AV) or acetosyringone (AS) intensified this process, i.e. transformation was more extensive than in the experiments omitting mediators. Among the products, isovanillic (IA) and vanillic (VA) acids were identified.

### INTRODUCTION

Fungal laccases (benzenediol:oxygen oxireductases, EC 1.10.3.2) are a multicoper inducible oxidases reducing oxygen to water and simultaneously performing one-electron oxidation of aromatic hydrogen donors (Leonowicz *et al.*, 2001; Mayer and Staples, 2002; Youn *et al.*, 1995). Because of their capability in oxidation of phenols they are receiving increasing interest as potential industrial enzymes in various applications such as pulp bleaching processes (Bourbonnais *et al.*, 1997a, 1997b and 1998; Li *et al.*, 1997), delignification (Leonowicz and Trojanowski, 1975), wood fiber modification (Leonowicz *et al.*, 2001) or remediation of contaminated water (Leonowicz *et al.*, 1997b). They have however a limited effect on lignin degradation due to their oxidative specificity (Leonowicz *et al.*, 1999; Szklarz and Leonowicz, 1986). Moreover, the measurement of pore sizes in wood shows that large molecules such as enzymes (*e.g.* laccase) does limits the penetration of the undegraded plant cell wall except where the wood cell wall is already partially decayed (Evans *et al.*, 1994; Fournoy *et al.*, 1991). Therefore, in recent years research has been focused on such potential low-molecular mass mediators of internal or external origin, which poses high enough redox potentials (>900 mV) to attack lignin and can migrate from the enzymes into tight ligno-

cellulose complex. Many possible low-molecular mass compounds have been suggested as candidates for a mobile factor to permeate wood cell walls and indicate decay. Some of these, such as veratryl alcohol, oxalate, malate, fumarate, and 3-hydroxyanthranilic acid, are produced as a result of fungal metabolism and their secretion enables the fungi to colonize and degrade the wood cell structure more effectively than other organisms (Eggert *et al.*, 1996; Hofrichter *et al.*, 1998; Lundquist and Kirk, 1978; Potthast *et al.*, 1999; Traquair, 1987). Most of the fungal enzymes are always located within the extracellular polysaccharide sheath surrounding the hyphae i.e. at the cell wall surfaces. The mediators are capable of diffusing from enzymes into the middle of wood cell wall structure and open up the pore size in wood what allow enzymes to penetrate and complete the degradative processes (Evans *et al.*, 1994; Call and Mücke, 1997).

It was also found that delignification of kraft pulp by laccase can be supported by some external (i.e. non-produced by fungi and absent in pulp) low molecular dyes or other aromatic hydrogen donors as acting mediators such as 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid - ABTS) (Bourbonnais *et al.*, 1997a; 1997b and 1998) or 1-hydroxybenzotriazole-HBT) (Bourbonnais *et al.*, 1998; Call, 1994; Srebotnik *et al.*, 1988; Srebotnik and Hammel, 2000).

This study was performed to investigate the laccase and mediator co-operation in modelling lignin dimer veratrylglycerol- $\beta$ -vanillate ether (VVE) oxidation. Earlier authors stated that constitutive laccase isolated from *Cerrena unicolor* can cooperate with acetovanillone (AV) and acetosyringone (AS) in degradation of high molecular fraction of lignosulfonates (Leonowicz *et al.*, 2001). The fungus *C. unicolor* is known as a very

<sup>1</sup> Wood and Paper Science, Chungbuk National University, Cheongju 361-763, Korea

<sup>2</sup> Department of Biochemistry, Maria Curie-Skłodowska University, Lublin PL 20031, Poland

<sup>3</sup> Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, Los Angeles, CA 90089, USA

\* Corresponding author (E-mail: ohga@forest.kyushu-u.ac.jp)

good producer of cheap constitutive laccase (Gianfreda *et al.*, 1998; Leonowicz *et al.*, 1997a and 1997b). Its cultivation for this reason does not require stimulation by the expensive and toxic for environment compounds such as 2,5-xylidine (Leonowicz *et al.*, 1997b). The present study is to examine capability of *C. unicolor* laccase to oxidize VVE, which is a non-phenolic lignin model compound with external mediators, HBT, AV and AS in order to choose one, most efficient system.

## MATERIALS AND METHODS

### Strain and culture conditions

*Cerreña unicolor* (Bull. ex Fr.) Murr., Einfarbige Tramete Strain T 143 was kindly provided by the Collection Molitoris, Botanic Institute, University of Regensburg. The fungus was maintained in 2% (w/v) malt agar slants. As an inoculum, pieces of agar were grown in the Lindenberg and Holm (1952) medium in non-agitated conical flasks for 7 days at 28 °C. The mycelial mats were subsequently collected and homogenized in a Warring blender.

The fermenter scale cultivation was performed at 28 °C in a 2.5 l Bioflo III (New Brunswick) fermenter containing 2 l of the Lindenberg and Holm (1952) medium. The fermenter was inoculated with crumbled fungal mats (10% total volume), aerated by 1 l air per minute and stirred at 300 rpm. Antifoam 289 emulsion (Sigma, St. Louis, USA) was occasionally added to the fermenter cultures.

### Reagents

Veratrylglycerol- $\beta$ -vanillate ether (VVE) was synthesized and kindly supplied by Dr. Atsumi Nishida; agar was from Junsei Chemical Co. (Tokyo, Japan); malt and yeast extracts were from DIFCO (Milwaukee, Wi. U.S.A.); syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehydazine), acetovanillone (AV, 4-hydroxy-3-methoxyacetophenone), acetosyringone (AS, 3,5-dimethoxy-4-hydroxyacetophenone), HBT (1-hydroxybenzotriazole),  $\alpha$ -methyl mannoside, and bovine liver catalase were from Aldrich-Chemie (Steinheim, Germany); bovine serum albumin and isovanillic acid (IA) were from Sigma (St. Louis, Mo. U.S.A.); AH-Sepharose-4B, DEAE-Cellulose and Sephadex G-50 'fine' were from Pharmacia (Uppsala, Sweden); syringaldehyde and vanillic acid (VA) were from Fluka A.G. (Buchs, Switzerland).

### Determination of protein

The protein content both in the culture fluids and in laccase preparations was determined according to Bradford (1976). Bovine albumin was used as a standard.

### Laccase purification

For the enzyme purification the method of Leonowicz *et al.* (1997a) with some our and Gianfreda *et al.* (1998) modifications was applied as follows. The aerated cultures at the tops of laccase activity were filtered

through Miracloth (Calbiochem, Lucerne, Switzerland). Each filtrate was desalted on the Sephadex G-50 column. The enzyme solutions were concentrated to ca. one tenth of the volume at 4 °C with the Amicon ultrafiltration system equipped with a filter type PTGC (cut off 10kDa). Then 50 ml portions of enzyme preparations were applied onto DEAE-Cellulose column (25×1.5 cm) preequilibrated with 5 mM TRIS/HCL buffer (pH 6.0). The fractions showing the highest laccase activity were poured onto a column of AH-Sepharose 4B coupled with syringaldehyde column (25×1.5 cm) equilibrated with 0.01 M citrate-phosphate buffer pH 5.0 prepared according to McIlvaine (1921). The fractions bounded with the carrier were removed by using 0.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and stored freeze dried. The enzyme activity both in the culture fluids and in laccase preparations was determined spectrophotometrically at 525 nm using syringaldazine on a Shimadzu UV-160 spectrophotometer according to Leonowicz and Grzywnowicz (1981). The activity was expressed in nkat per litre. To exclude endogenous peroxide, the 10 min. preincubation (stirring) of the enzyme sample with catalase (10 mg/ml), was performed. No lignin peroxidase activity was found in the enzyme preparation. (Tien and Kirk, 1988).

### Enzyme reaction

To a flask with 5 ml of 0.1 M McIlvaine buffer pH 5.3 laccase (300 nkat) was added, VVE (50  $\mu$ l of 10 mM), HBT, AV or AS, each 50  $\mu$ l of 15  $\mu$ M. The flasks were rotary shaken (180 rpm) at 28 °C. Control experiments lacked either laccase or mediators in the reaction mixture. The reaction products after alkalization with 1 M NaOH (to melt the sediment) were extracted twice with one volume of diethyl ether, evaporated under IR lamp and redissolved in 50% ethanol.

### Thin-layer chromatography

Extracts were evaporated to small volumes and analyzed by TLC on silica-gel plates (DC-Alurolle Kieselgel 60 F 254 from Merck) according to Leonowicz *et al.* (1984) using the solvent system benzene : methanol : propionic acid (88:8:4). The phenolics were visualized with diazosulfamide reagent according to the procedure described by Leonowicz *et al.* (1968).

### Capillary electrophoresis

Micellar electrokinetic chromatography (MEKC) analyses were performed on Spectra 100 Thermo Separation Products (San Jose, USA). The separations were carried out using fused silica capillary with a total length of 69 cm (44 cm to detection window) and an inner diameter of 50  $\mu$ m. Applied voltage was 29 kV and the capillary temperature was maintained at 25 °C. Injection was done in the hydrodynamic mode for 0.5 s. Detection was performed by measuring absorbance at 210 nm. Buffer solution used was 100 mM boric acid with 100 mM SDS at pH 8.8 (adjusted with NaOH). All samples, buffer solutions, and conditionings before use were filtered through 0.22  $\mu$ m filters.

## RESULTS AND DISCUSSION

**Laccase isolation and purification**

The *C. unicolor* extracellular laccase was isolated and purified from aerated fermenter culture. The final enzyme preparation was purified more than 32-fold (Table 1). No lignin peroxidase activity was found in the enzyme preparation (Tien and Kirk 1988). It seems that our laccase using 4 steps of purification was sufficiently purified to carry out the investigations whose are presented in this paper.

**Transformation of substrate (VVE, veratrylglycol- $\beta$ -vanillate ether) by laccase and laccase/mediator couples**

Transformations of VVE by laccase alone and laccase/mediator systems (Mediators chemical structures Fig. 1) are different. The results shown in Fig. 2 confirm much more efficient reaction with laccase/mediator systems than with laccase alone. The transformation efficiency (mediating activity) of laccase/AV and laccase/AS

systems (Fig. 2) was comparable to laccase/HBT system and even better in the case of laccase/AS than laccase/HBT. This result shows that AV and AS can work as laccase mediators.

**Mediation efficiency**

Studying the VVE depolymerization process further we examined the products of mediating activity. When laccase and the mediators were incubated with VVE depolymerization occurred. The resulted vanillic acid (VA) and isovanillic acid (IA) were detected both chromatographically (Table 2) and by capillary electrophoresis (Figs. 3, 4 and 5). The control map (without laccase) of capillary electrophoresis of substrate, mediators and products is presented in Fig. 3.

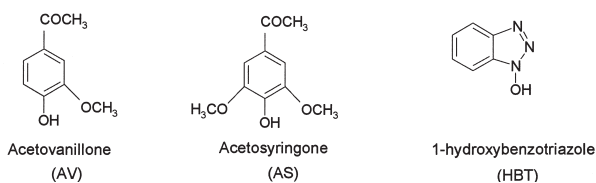
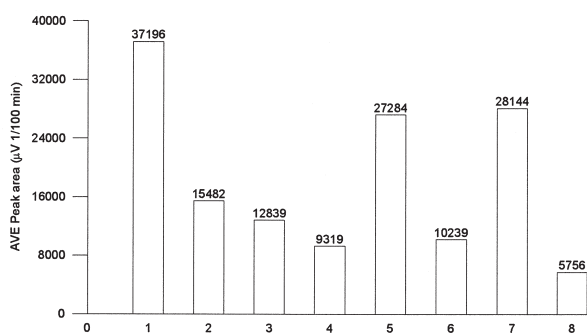
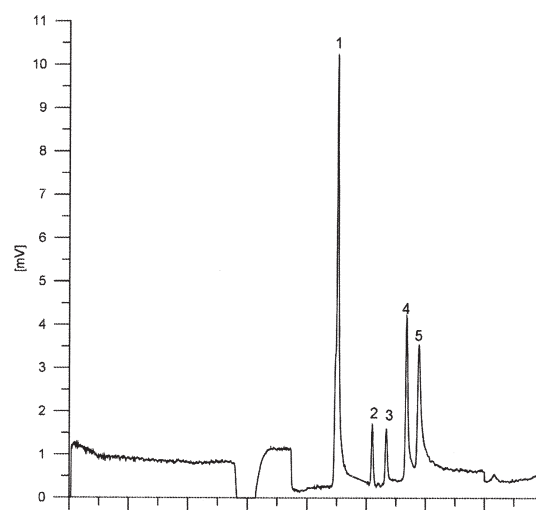
As shown in Table 2 and illustrated in Figs. 4, 5 and 6 with reference to control map (Fig. 3) laccase and laccase/mediator systems partly transform the substrate VVE into IA and VA. No significant difference was observed between 6 h and 12 h incubation. At the moment although we do not have an reliable mechanistic

**Table 1.** Isolation and purification of *C. unicolor* laccase

Purification step	Total activity (nkat/l)	Specific activity (nkat/mg)	Yield (%)	Purification (fold)
Culture filterates	87458	436.83	100.00	1.00
Sephadex G-50	80147	520.44	91.64	1.19
Ultrafiltration	69785	2791.40	79.79	6.39
DEAE-Cellulose	45874	11468.50	52.45	26.25
Syngyl-AH-Sepharose 4B	29874	14225.71	34.16	32.57

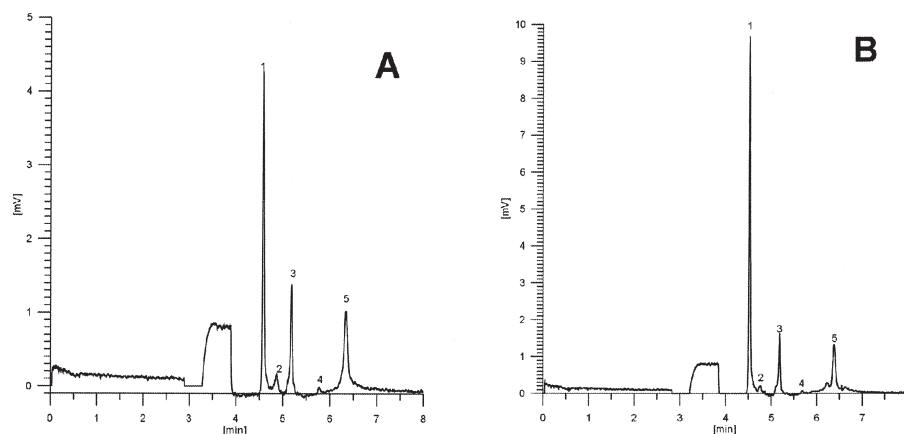
**Table 2.** Aromatic acids produced from VVE detected by thin layer chromatography

Aromatic acid	Rf of commercial compounds	Rf of reaction products
Isovanillic acid (IA)	0.5912	0.5898
Vanillic acid (VA)	0.6759	0.6777

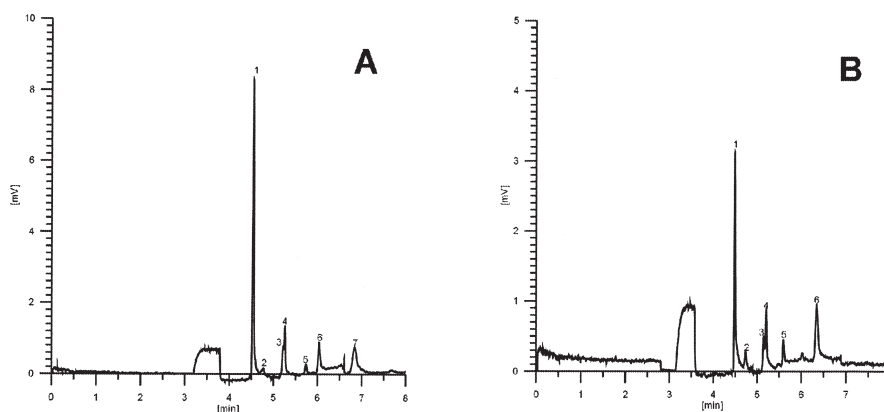
**Fig. 1.** Chemical structure of used mediators.**Fig. 2.** VVE (veratrylglycol- $\beta$ -vanillate ether) transformations by laccase, and laccase/mediator systems. 1=laccase+VVE after 6 h incubation; 2=12 h; 3=laccase/HBT+VVE after 6 h; 4=12 h; 5=laccase/AV (acetovanillone)+VVE, 6h; 6=12 h; 7= laccase/AS (acetosyringone)+VVE, 6h; 8=12 h.**Fig. 3.** Capillary electrophoresis of substrate, mediators and products (control map): 1=VVE; 2=IA (isovanillic acid); 3=VA (vanillic acid); 4=AV; 5=AS. All abbreviations refer to Fig. 2.

explanation for this result, tentative VVE degradation scheme could be proposed as shown in Fig. 7 based on the capillary electrophoresis results by the laccase/mediator systems. A similar observation has previously been reported by Li *et al.* (1997) studying the degradation of a lignin  $\beta$ -0-4 dimer, 1-(3,4-dimethoxy)propan-1,3-diol. The presented results in this report show that

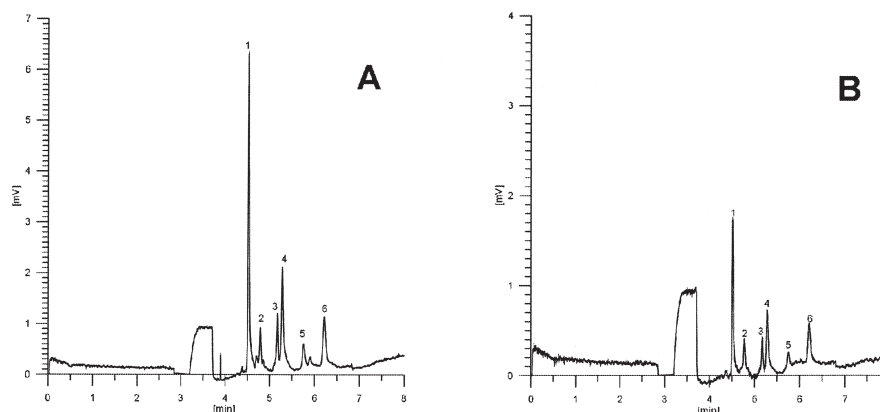
some laccase is able for transformation lignin model dimer and that AV and AS stimulated the process. As can be seen in Fig. 2, AS was even more efficient than HBT. As well AV as AS (contrary to already known mediators, like HBT or ABTS), are produced in the processes of wood transformation, have less complicated formulae and are cheaper than ABTS or HBT. The sources



**Fig. 4.** Capillary electrophoresis of laccase reaction on VVE: A—after 6 h incubation; B—after 12 h: 1=VVE; 2=unknown; 3=IA; 4=VA; 5=unknown. All abbreviations refer to Fig. 2.



**Fig. 5.** Capillary electrophoresis of laccase and laccase/acetovanillone reaction on VVE: A—after 6 h incubation; B—after 12 h: 1=VVE; 2=unknown; 3=IA; 4=VA; 5=AV; 6 and 7=unknown. All abbreviations refer to Fig. 2.



**Fig. 6.** Capillary electrophoresis of laccase and laccase/acetosyringone reaction on VVE: A—after 6 h incubation; B—after 12 h: 1=VVE; 2=unknown; 3=IA; 4=VA; 5=AS; 6=unknown. All abbreviations refer to Fig. 2.



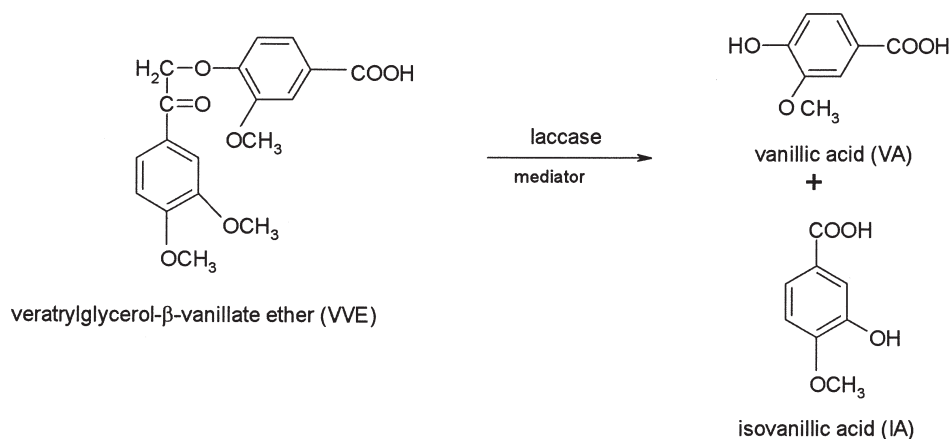


Fig. 7. Possible route of VVE transformation by laccase/mediator systems.

of lignocellulose that occur in various forms in nature (mainly as wood) are so vast that they can only be compared to those of water (Leonowicz *et al.*, 2001). The results presented in this report show that laccase with using of some compounds as mediators is able for degradation of dimer being a model compound of lignin, which is the most undegradable constituent of lignocellulose complex. It may be one more (biotechnological) system for the bleaching of kraft pulps, in combination with oxygen delignification techniques.

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