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Oxidation of isoeugenol and coniferyl alcohol catalyzed by laccases isolated from *Rhus vernicifera* Stokes and *Pycnoporus coccineus*

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Abstract

Laccases isolated from *Rhus vernicifera* Stokes (tree) and *Pycnoporus coccineus* (fungus) catalyzed the oxidation of isoeugenol (**1**) and coniferyl alcohol (**5**) in acetone–water (1:1, v/v). These oxidations follow a first order rate law. In general, the rates of *Pycnoporus* laccase-catalyzed oxidation of **1** and **5** are approximately three and seven times faster than the corresponding rates of *Rhus* laccase-catalyzed oxidation, respectively. Thus, synthesis for 2-(4-hydroxyphenyl)coumaran type compounds, such as dehydrodiconiferyl alcohol, can be accomplished by *Rhus* laccase-catalyzed dehydrogenative polymerization of the corresponding 1-(4-hydroxyphenyl)–1-propene derivatives. The reaction proceeds under very mild reaction conditions. The resulting reaction mixtures are chromatographed on a silica gel column to isolate the products in approximately 30–40% yield.

Keywords: *Rhus* laccase; Enzyme-catalyzed oxidation; Isoeugenol; Coniferyl alcohol

1. Introduction

The enzyme laccase (EC 1.10.3.2) occurs as a major enzyme in wood decaying mushrooms [1,2], differentiating xylems of trees [3–6], and in the exudates from the lacquer tree and similar species [1,7]. When tissue of Chinese or Japanese lacquer tree (*Rhus vernicifera* Stokes) is injured, it produces an exudate that contains urushiols, laccase, stellacyanin and peroxidase. On treatment with acetone at room temperature, most of the constituents of the exudate are dissolved into acetone, giving approximately 10% of acetone insoluble material (acetone powder) that contains approximately 0.2–0.5% of *Rhus* laccase by weight [7–11]. Pure *Rhus* laccase, usually obtained from the acetone powder according to the modified procedure of Reinhammer [7], is intensively investigated by several authors [1,7–12]. It is a glycoprotein containing four copper atoms and has a relative molecular mass of approximately 100,000 Da as

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determined by SDS/polyacrylamide gel electrophoresis of the purified enzyme [12]. This laccase is capable of catalyzing single-electron-transfer oxidations of catechol derivatives, such as urushiols, with dioxygen. At the same time, one equivalent of dioxygen is reduced to two equivalents of water.

Recently, *Rhus* laccase was found to catalyze oxidation of 1-(4-hydroxyphenyl)–1-propene derivatives, such as isoeugenol (**1**) and coniferyl alcohol (**5**), with dioxygen that was in turn reduced into water by a four-electron process [11 12]. The products of these reactions are mostly dimeric products. In contrast, laccase isolated from the white-rot fungus *Pycnoporus coccineus* [13] readily catalyzed the oxidation of both **1** and **5** with dioxygen that produces mostly the corresponding dehydrogenative polymerization products (DHPs) with trace amounts of dimeric products. Thus, the objectives of this investigation are twofold: (a) to study the kinetics of the oxidation of **1** and **5** with dioxygen catalyzed by *Rhus* and *Pycnoporus* laccases, and (b) to explore the potential of applying these reactions in synthesis of 2-(4-hydroxyphenyl)coumaran derivatives from the corresponding 1-(4-hydroxyphenyl)–1-propene compounds.

2. Results and discussion

Rhus laccase was purified from acetone powder using the procedure of Reinhammer with minor modification. The purified enzyme gave one band at M_r 100,000 on SDS/polyacrylamide gel electrophoresis. The *Rhus* laccase is rather stable at alkaline pH in phosphate buffer ($Na_2HPO_4/NaH_2PO_4/NaOH$) solution. The activity of *Rhus* laccase towards laccase-catalyzed oxidation of **1** and **5** was found to be optimum at the pH range of 8.5–9.0 (Fig. 1). At the optimum pH, the catalytic activity of *Rhus* laccase towards the oxidation of **1** with dioxygen is approximately three times higher than that towards the oxidation of **5**. In contrast,

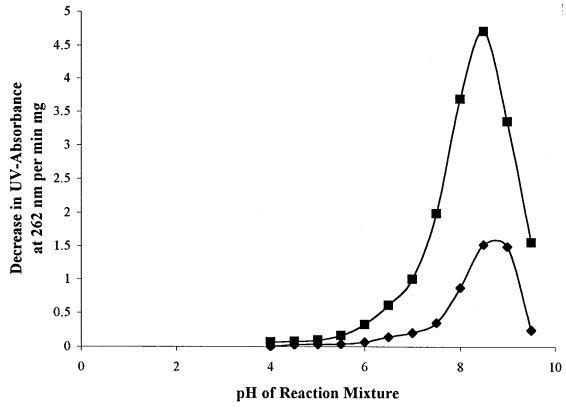

Fig. 1. Effects of pH on the laccase activity of *Rhus* laccase using **1** and **5** as substrates; a solution of 0.12 mM substrate in a 0.04 M Na_2HPO_4/NaH_2PO_4 buffer solution at 30°C with purified *Rhus* laccasse activity of 5 units/50 ml reaction mixture. Isoeugenol -■-■-, coniferyl alcohol -◆-◆-.

Pycnoporus laccase has the optimum pH range of 3.5–4.5 for the oxidation of these substrates with similar catalytic activity of *Pycnoporus* laccase towards both **1** and **5** with dioxygen (Fig. 2).

In the buffer solution, the catalytic activities of *Rhus* laccase in the oxidation of **1** and **5** at pH 7 are 21.4% and 13.3% of the corresponding optimum values at the optimum pH around 8.5, respectively. Thus, ratio of the decrease in UV-absorbance at 262 nm/min mg at pH 7–8.5 are 1.006–4.71 and 0.206–1.55 for **1** and **5**, respectively (Fig. 1). The catalytic activities of *Rhus* laccase towards the oxidation of **1** and **5** in deionized water (pH 7) are much less active than that in the buffer solution at pH 7. The decreases in UV-absorbance at 262 nm/min mg for **1** and **5** in water (pH 7) are only 0.31 and 0.065, respectively, approximately 31% of the corresponding values in the buffer solution at pH 7. The relative catalytic activity of *Rhus* laccase towards **1** and **5** in aqueous solution does not decrease at neutral pH when hydrophilic organic solvents, such as methanol and acetone, are added to the reaction medium up to 30% except for **1** in acetone–water system (Fig. 3). When methanol content is 20% in methanol–water system, the relative catalytic

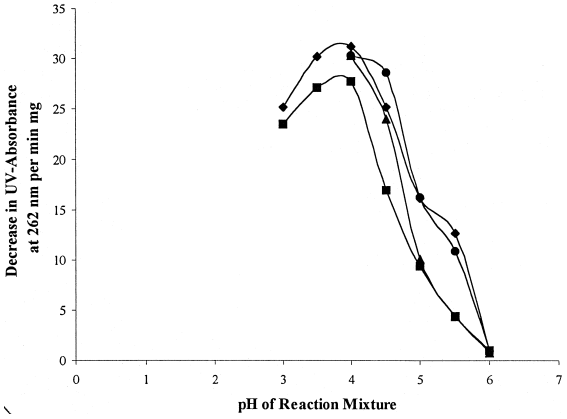

Fig. 2. Effects of pH on the laccase activity of *Pycnoporus* laccase using **1** and **5** as substrates; a solution of 0.12 mM substrate in a 0.04 M buffer solution at 30°C with *Pycnoporus* laccasse activity of 30 units/50 ml reaction mixture. Buffer solutions: Citric acid/Na_2HPO_4 (pH 3.0–6.5), Isoeugenol -■-■-, coniferyl alcohol -◆-◆-; AcONa/AcOH (pH 4.0–5.4), isoeugenol -●-●-, coniferyl alcohol -▲-▲-.

activity of *Rhus* laccase towards **1** increases approximately 6%. When the acetone content is 30% in acetone–water system, the relative catalytic activity of *Rhus* laccase towards **1** decreases to approximately 85% of the original. The relative activity of *Rhus* laccase towards **1** and **5** then decreases sharply to approximately 30–40% of the original, when the organic solvent content is 40% (Fig. 3). When the ratio of organic solvent content is 50% in the organic solvent–water system, the relative catalytic activity of *Rhus* laccase towards **1** and **5** is less than 15% of the original activity. Here, the relative catalytic activity of *Rhus* laccase is as percentage of the decrease in UV-absorbance at 262 nm/min mg for **1** in water (pH 7), which is 0.31.

The kinetic studies of the *Rhus* and *Pycnoporus* laccase-catalyzed oxidation of **1** and **5** were carried out in the acetone–water mixture at neutral pH and temperature range of 25–30°C to simplify the procedure. The *Rhus* laccase catalyzed rather slowly the oxidation of substrates **1** and **5** with dioxygen. In contrast, *Pycnoporus* laccase readily catalyzed the oxidation of substrates **1** and **5** by dioxygen to produce mostly the corresponding DHP (Fig. 4). With a reaction time of 24 h at 30°C, the *Rhus* laccase-catalyzed oxidation of **1** with dioxygen resulted in formation of approximately 43 mol% of dehydrodiisoeugenol (**2**), 12 mol% of *threo*-2-[4-(propren-1-yl)guaiacoxyl)]-1-guaiacyl-1-propanol (**3a**), 3 mol% of its *erythro* isomer, *erythro*-2-[4-(propren-1-yl)guaiacoxyl]-1-guaiacyl-1-propanol (**3b**), 2 mol% of tetrameric product **4** and some oligomers but no DHP. The constituents of the oligomeric mixture were not identified. In contrast, when the same reaction was carried out by using *Pycnoporus* laccase as catalyst, approximately 95 mol% of the substrate was converted into oligomers and DHPs, the latter being the major products, but no dimers (Table 1).

The oxidation of **5** catalyzed by *Rhus* laccase is even slower. With a reaction time of 72 h at 30°C, the oxidation produced approximately 31 mol% of dehydrodiconiferyl alcohol (**6**), 11 mol% of a mixture of *threo*-guaiacylglycerol-β-coniferyl ethers (**7a**) and *erythro*-guaiacylglycerol-β-coniferyl ethers (**7b**) and 18 mol% of pinoresinol (**8**), but no oligomers and DHP (Ta-

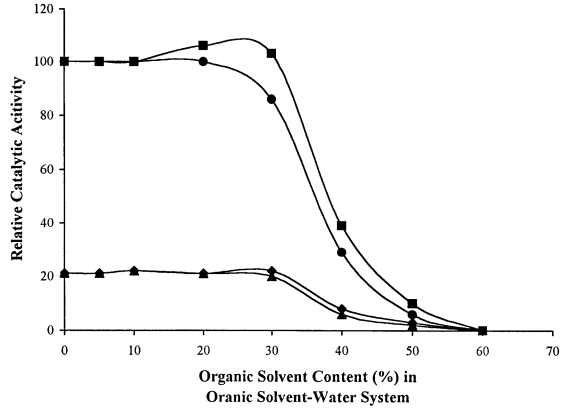

Fig. 3. The effect of hydrophilic organic solvents on the activity of *Rhus* laccase in water–organic solvent systems at 30°C using **1** and **5** as substrates. The values are relative to the decrease in UV-absorbance at 262 nm/min mg for **1** in water (pH 7) 0.31 as 100%. In water–acetone: Isoeugenol -■-■-, coniferyl alcohol -◆-◆-. In methanol–water: Isoeugenol -●-●-, coniferyl alcohol -▲-▲-.

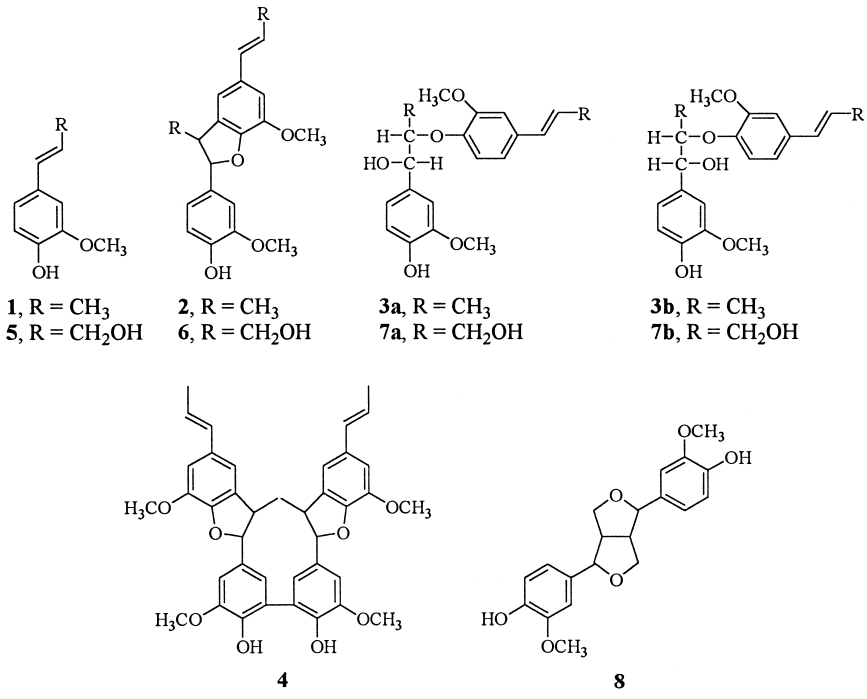

Fig. 4. Dimeric and tetrameric DHPs from laccase-catalyzed oxidation of **1** and **5** with dioxygen (reaction conditions, see Tables 1 and 2).

ble 2). When acetone-powder was used instead of the purified *Rhus* laccase as catalyst for the oxidation of **1** and **5**, the constituents of the resulting reaction mixtures did not change appreciably. Only dimeric products **7a** and **7b** were absent in the oxidation of **5**.

The kinetic studies of the reactions show that the rate of disappearance of the substrate follows a pseudo-first order kinetics with respect to substrate. The rate constants for the disappearance of **1** and **5** were determined to be 2.33×10^{-5} and 5.0×10^{-6} s^{-1} for the *Rhus*

Table 1
Yields of dimeric and polymeric products from laccase-catalyzed oxidation of **1** in 20 ml acetone–water mixture (1:1, v/v) at 23–30°C

Substrate (**1**) used, mg (mmol)	Laccase activity used	Reaction temp. (°C)	Reaction time (h)	Yield of products (mol%)						
				2	**3a**	**3b**	**4**	Oligo.	DHP-1	DHP-2
Crude laccase from R. vernicifera Stokes (acetone-powder); laccase activity 1.0×10^4 *units / g*										
507 (3.09)	1.0×10^4	26.0	2	41.4	19.0	4.7	2.1	19.3	–	–
508 (3.10)	1.0×10^4	31.0	24	43.1	17.6	4.3	3.3	18.9	–	–
503 (3.07)	2.0×10^4	30.0	2	41.1	18.5	4.8	1.1	16.9	–	–
Purified laccase from R. vernicifera Stokes (aqueous solution); laccase activity 1.7×10^3 *units / ml*										
506 (3.09)	1.7×10^3	23.0	24	43.0	11.7	3.3	1.7	19.7	–	–
Laccase from P. coccineus (aqueous solution); laccase activity 2.7×10^5 *units / ml*										
507 (3.09)	1.35×10^4	29.0	24	–	–	–	–	13.0	62.1	20.9
508 (3.10)	1.70×10^4	24.5	24	–	–	–	–	9.2	60.6	25.5

Table 2
Yields of dimeric and polymeric products from laccase-catalyzed oxidation of **5** in 20 ml acetone–water mixture (1:1, v/v) at 25–31°C

Substrate (**1**) used, mg (mmol)	Laccase activity used	Reaction temp. (°C)	Reaction time (h)	Yield of products (mol%)			
				6	**7a** and **7b**	**8**	DHP
Crude Laccase from R. vernicifera Stokes (acetone–powder); laccase activity 1.0×10^4 units / g							
516 (2.87)	1.0×10^4	25.0	72	28.7	–	12.1	–
521 (2.89)	1.0×10^4	30.0	72	29.2	–	15.6	–
Purified laccase from R. vernicifera Stokes (aqueous solution); laccase activity 1.7×10^3 units / ml							
501 (2.78)	1.7×10^3	30.0	2	–	–	–	–
503 (2.79)	1.7×10^3	31.0	72	30.5	10.6	18.6	–
Laccase from P. coccineus (aqueous solution); laccase activity 2.7×10^5 units / ml							
501 (2.78)	1.35×10^4	30.0	2	8.9	–	23.2	–
500 (2.78)	1.35×10^4	30.0	12	–	–	Trace	98.1
508 (3.10)	1.35×10^4	30.0	72	–	–	1.8	96.0

laccase-catalyzed oxidation with dioxygen under same reaction condition in acetone–water mixture (1:1, v/v) at 25°C, respectively (Fig. 5). Thus, the former is approximately 4.7 times faster than the latter. Similarly, the rate constants for the disappearance of **1** and **5** were determined to be 7.33×10^{-5} and 3.5×10^{-5} s^{-1} for the *Pycnoporus* laccase-catalyzed oxidation under the same reaction condition in acetone–water mixture (1:1, v/v) at 25°C, respectively (Fig. 6). Here again, the former is approximately two times faster than the latter. Thus, the rate for the *Rhus* laccase-catalyzed oxidation of monolignols with dioxygen is, in general, approximately 3–7 times slower than that for the corresponding *Pycnoporus* laccase-catalyzed oxidation with the rate being dependent on the nature of monolignol. The slower rates for the *Rhus* laccase-catalyzed oxidations are probably due to differences in the nature and molecular mass of these glycoproteins, as well as the ligand binding mode of type 2 and type 3 copper sites [8–10].

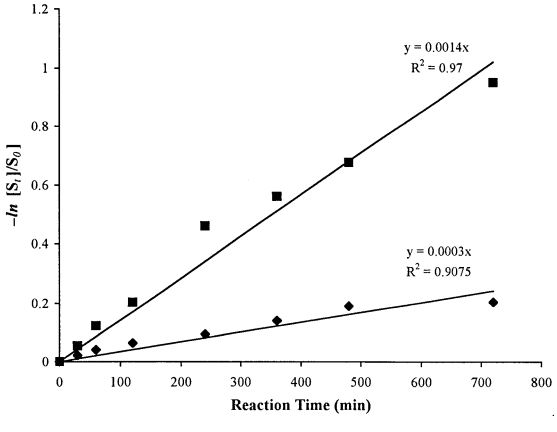

Fig. 5. Pseudo-first order kinetics for *Rhus* laccase-catalyzed oxidation of **1** and **5** with dioxygen, $-\ln[S_t]/[S_0]$ versus reaction time (min); 3.05 mmol of the substrate in 20 ml acetone–water mixture (1:1, v/v) at 25°C with purified *Rhus* laccase activity of 1.7×10^3 units. Isoeugenol -■-■-; coniferyl alcohol -◆-◆-.

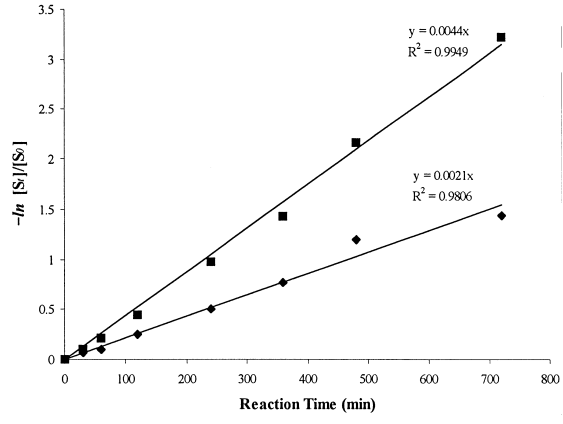

Fig. 6. Pseudo-first order kinetics for *Pycnoporus* laccase-catalyzed oxidation of **1** and **5** with dioxygen, $-\ln[S_t]/[S_0]$ versus reaction time (min); 1.39 mmol of the substrate in 20 ml acetone–water mixture (1:1, v/v) at 25°C with *Pycnoporus* laccase activity of 1.35×10^4 units. Isoeugenol -■-■-; coniferyl alcohol -◆-◆-.

All these characteristics indicate that the *Rhus* laccase-catalyzed oxidation of 1-(4-hydroxyphenyl)−1-propene derivatives is suitable for use in simplifying syntheses of the corresponding 2-(4-hydroxyphenyl)coumaran derivatives, which are usually complex. For example, synthesis of **6** from 4-*O*-benzylvanillin and methyl 2-benzyloxy-3-methoxy-5-(dimethoxy-methyl) phenyl acetate via aldol addition requires a total of 12 steps with an overall yield of less than 5 mol% per starting materials [14]. This was simplified by the application of the *Rhus* laccase-catalyzed oxidation of **5** in acetone−water mixture under the optimum condition. After removal of acetone from the reaction mixture under reduced pressure, the resulting solution was freeze-dried to recover all products as resinous residue. The residue was then chromatographed on a silica gel column to isolate individual products. The yield of **6** was approximately 30 mol% in the *Rhus* laccase-catalyzed oxidation of **5**. In contrast, *Pycnoporus* laccase readily catalyzed the oxidation of **5** to produce the corresponding DHP [11,12]. With the same enzymatic method, **2** can be synthesized with an overall yield of approximately 40 mol% with a reaction time of 24 h at 25°C.

This research group is still investigating the reaction mechanisms and the factors contributing to the difference in the disappearance rate for the substrates in the fungal and tree laccase-catalyzed oxidation. However, it is likely that the differences are effected by difference in the nature and molecular mass of glycoproteins as well as the ligand binding mode of the trinuclear cluster site comprising of T2 and T3 coppers. Solomon et al. [10] have postulated on the basis of available kinetic and spectroscopic date that the fully reduced laccase would be oxidized to a hydroperoxide intermediate by dioxygen in the presence of water in the laccase-catalyzed oxidation. Accordingly, a fully reduced laccase contains four Cu(I) ions. The hydroperoxide intermediate would be then converted into native laccase intermediate by transferring an electron each from T1 and T2 with a total of two electrons to the −O−O− bond in the hydroperoxyl group. In the presence of hydrogen ions, this would result in the cleavage of the hydroperoxyl group with elimination of a mole of water to give the native laccase intermediate with two hydroxyl ligands bonded to T2–$T3_a$ and $T3_a$–$T3_b$. A phenol substrate would reduce the Cu(II) in T1 to Cu(I) in the native laccase intermediate with a concomitant hydrogen abstraction from the substrate. At the same time, a mole of water would be eliminated from one of the two hydroxyl ligands in the trinuclear cluster site with a transfer of an electron from either Cu(I) in T1 to Cu(II) in T2 or Cu(II) in $T3_b$. Thus, the phenol substrate would be oxidized to produce the corresponding phenoxyl radical. This initial step would facilitate the laccase-catalyzed cycle of transferring electrons from the phenolic substrate to dioxygen in four single electron-transferring steps. In each cycle, 1 mol

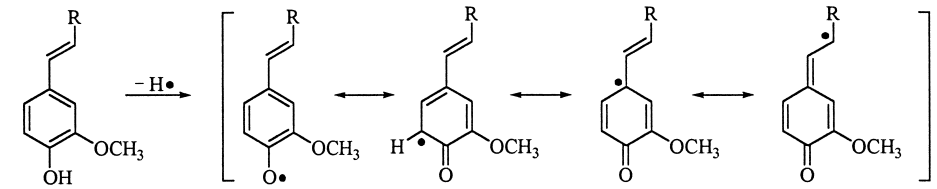

Fig. 7. Resonating mesomers of the phenoxyl radicals **1a** and **5a** that are produced from laccase-catalyzed oxidation of **1** and **5** with dioxygen, respectively.

of dioxygen is reduced to 2 mol of water according to the stoichiometry, $O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$. The phenolic substrate would also be probably attached to the enzyme through ligand binding during this process.

On laccase-catalyzed hydrogen-abstraction with dioxygen, the resulting phenoxyl radicals from either **1** or **5** are resonating among their mesomers **1a**–**1d** or **5a**–**5d**, respectively (Fig. 7). **2** results from the recombination between radical species **1b** and **1d** and subsequent cyclization of the resulting β-5′ quinone–methide intermediate via intramolecular nucleophilic addition. In addition, **3a** and **3b** result from the recombination between radical species **1a** and **1d** in equal mole equivalent and subsequent nucleophilic addition of water on C-α of the resulting quinone–methide intermediate. The tetrameric product **4** produced by dehydrogenative dimerization of **2**. Similarly, **6** is produced from the recombination of radical species **5b** and **5d** in equal mole equivalent, while **7a** and **7b** are formed from that of radical species **5a** and **5d**. **8** is produced by the recombination of 2 moles of **5d** to the β–β′ quinone–methide intermediate, followed by cyclization via intramolecular nucleophilic addition.

3. Conclusion

Laccases isolated from *R. vernicifera* Stokes (tree) and *P. coccineus* (fungus) catalyzed oxidation of **1** and **5**. The oxidation follows pseudo-first order kinetics. For the *Rhus* laccase-catalyzed oxidation with dioxygen, the rate constants for the disappearance of **1** and **5** were determined to be 2.33×10^{-5} and 5.0×10^{-6} s^{-1} under the same reaction condition in acetone–water mixture (1:1, v/v) at 25°C, respectively. The oxidation rate of **1** is approximately 4.7 times faster than that of **5**. For the *Pycnoporus* laccase-catalyzed oxidation, the rate constants for the disappearance of **1** and **5** were determined to be 7.33×10^{-5} and 3.5×10^{-5} s^{-1} under the same reaction condition in acetone–water mixture (1:1, v/v) at 25°C, respectively. The oxidation rate of **1** is approximately two times faster than that of **5**. Moreover, for the oxidation of **1** and **5**, the rate of *Pycnoporus* laccase-catalyzed oxidation is approximately three and seven times faster that of *Rhus* laccase-catalyzed oxidation, respectively. Thus, the rate for the *Rhus* laccase-catalyzed oxidation of monolignols with dioxygen is, in general, approximately 3–7 times slower than that for the corresponding *Pycnoporus* laccase-catalyzed oxidation with the rate being dependent on the nature of both monolignol and laccase.

Synthesis of 2-(4-hydroxyphenyl)coumaran type compounds, such as dehydrodiconiferyl alcohol, can be accomplished by *Rhus* laccase-catalyzed dehydrogenative dimerization of the corresponding 1-(4-hydroxyphenyl)–1-propene derivatives. The reaction proceeds under very mild reaction conditions. The resulting reaction mixtures are chromatographed on a silica gel column to isolate the products in approximately 30–40% yield. Acetone powder, acetone insoluble material of an exudate from *R. vernicifera* Stokes, is also very effective as catalyst instead of purified *Rhus* laccase in the reaction.

4. Experimental

4.1. Pycnoporus laccase

Laccase from *P. coccineus* was purchased from Koken, Tokyo, Japan. Laccase activity; 2.7×10^5 units/ml. The laccase activity was measured by syringyalazine assay method as modified by Grassin and Dubourdien [15]. One unit of laccase is defined as the amount of laccase activity oxidizing 1 nmol of syringyalazine/min in 0.1 M acetate buffer solution (pH 5) at 20°C.

4.2. Isolation of Rhus laccase

Laccase from *R. vernicifera* was isolated from the acetone powder obtained from Chinese

lacquer tree (*R. vernicifera* Stokes) according to the procedure of Reinhammer [7] with a minor modification. The acetone powder was suspended in 0.01 M potassium phosphate buffer solution (pH 6.0). The suspension was stirred for 8–12 h, then filtered. The filtrate was chromatographed on a CM-Sephadex C-50 column, then dialyzed with a cellulose membrane. The eluate was chromatographed on a DEAE-Sephadex A-50 column, then concentrated on a CM-Sephadex C-50 column. The resulting solution was finally desalted and concentrated with a CF25 membrane filtration. The purified laccase gave only one band with a relative mass of approximately 10,000 Da based on SDS/polyacrylamide gel electrophoresis. Laccase activity; 1.7×10^3 units/ml. The laccase was measured by syringyalazine assay method as modified by Grassin and Dubourdien [15]. One unit of laccase is defined as the amount of laccase activity oxidizing 1 nmol of syringyalazine/min in 0.1 M NaOAc buffer solution (pH 5) at 20°C.

4.3. Electrophoresis

SDS/polyacrylamide gel electrophoresis was performed on an ATTO system with a gel gradient of 5–20% acrylamide. Protein Mr standards were prestained SDS-PAGE standard: phosphoryase B (106,000), bovine serum albumin (80,000), ovalbumin (49,500), carbonic anhydrase (32,500), soybean trypsin inhibitor (27,500), and lysozyme (8500). Protein bands were stained with Coomassie blue.

4.4. Kinetic studies on the laccase-catalyzed oxidation of **1** and **5**

4.4.1. Effect of pH on catalytic activity of laccase towards substrate

To a solution of 0.12 mM substrate in an appropriate 0.04 M buffer solution was added 5 units of purified *Rhus* laccase or 30 units of *Pycnoporus* laccase/50 ml of reaction mixture a suitable amount of laccase at 30°C. The decrease in the UV-absorbance at 262 nm was then measured spectrophotometrically. The buffer solutions used were: (1) citric acid/Na_2HPO_4 for pH 3.0–6.5, (2) NaOAc/HOAc for pH 4.0–5.4, (3) Na_2HPO_4/NaH_2PO_4 for pH 6.0–85, and (4) $Na_2CO_3/NaHCO_3$ for pH 8.5–9.5.

4.4.2. General procedure for laccase catalyzed oxidation

A substrate (ca. 3 mmol) was dissolved in 10 ml of Me_2CO. To the resulting solution was added 10 ml of deionized H_2O, so that the ratio of Me_2CO to H_2O is 1:1 (v/v). To this mixture, an appropriate amount of laccase (see Tables 1 and 2) was added and the reaction mixture was kept at 25°C for 24 h using a thermostat and under mechanical stirring. Samples of the reaction mixture were taken at certain time intervals and extracted with CH_2Cl_2. The resulting solutions were then dried over anhydrous Na_2SO_4, and analyzed by gas chromatography (GC) and GCMS for substrates and oxidation products.

4.4.3. General procedure for GC

GC analysis was carried out with HP 5890 Series II gas chromatograph instrument fitted with a HP 5 column (cross-linked 5% diphenyl and 95% dimethyl polysiloxane; 30 m × 0.25 mm i.d., film thickness 0.25 μm). The samples were injected by the HP automatic injector into the injection port at 200°C. The initial oven temperature was 80°C and was held for 1 min, then raised at a rate of 20°C/min to 200°C and held for another 1 min. The oven temperature was again raised at the rate of 20°C/min to 280°C and held for 1 min. Signals were detected by a FID at the temperature of 240°C.

4.4.4. General procedure for GCMS analysis

GCMS analysis was carried out with a HP GCD Plus GC instrument equipped with a HP 5

column (cross-linked 5% diphenyl and 95% dimethyl polysiloxane; 30 m × 0.25 mm i.d., film thickness 0.25 μm). The samples were injected manually at 200°C. The initial oven temperature was 80°C and was held for 1 min, then raised at a rate of 20°C/min to 200°C and held for another 1 min. Finally, the oven temperature was raised again at the rate of 20°C/min to 280°C and hold for 1 min. The signals were detected by a FID at 240°C.

4.5. Isolation of dimeric and tetrameric products and DHP from the oxidation mixtures

The reaction mixture was extracted by shaking with $ClCH_2CH_2Cl$. The $ClCH_2CH_2Cl$–H_2O-insoluble material (DHP) was filtered and purified. The $ClCH_2CH_2Cl$-soluble part was chromatographed on a silica gel column (50 × 1.5 cm i.d.) using $CHCl_3$–CH_3OH (20:1, v/v) as eluent. The eluents were corrected every 10 ml and monitored by *tlc* to isolate individual dimeric and tetrameric products. The fractions with the same R_f were collected, and the solvent was removed under reduced pressure. The purity of residuals was monitored by GC. The almost pure fractions were recrystallized from an appropriate solvent as described below. The purified products were identified by *tlc*, ^1H and ^{13}C NMR spectroscopies. The compounds identified and their yields are given in Tables 1 and 2. The ^{13}C NMR data are given in Tables 3 and 4.

Table 3
^{13}C chemical shifts for carbons in **2**, **3a** and **3b** and β-5 type tetramer **4** isolated from the reaction mixture of *Rhus* laccase-catalyzed oxidation of **1**. Solvent: DMSO-d_6

Carbons	^{13}C chemical shifts of dimeric products (δ)			
	2	**3a** (*threo*)	**3b** (*erythro*)	**4**
C-α	92.3	74.8	74.0	91.9
C-β	44.2	78.6	78.8	44.4
C-γ	17.2	15.4	15.0	17.3
C-1	131.2	132.6	133.5	130.2
C-2	110.6	111.4	111.2	109.2
C-3	147.5	146.5	146.1	147.7
C-4	146.1	145.5	145.3	143.6
C-5	115.6	114.6	114.6	125.2
C-6	118.8	119.5	119.1	121.0
OCH_3	55.7	55.6	55.6	56.0
C-α′	130.2	131.0	130.6	131.0
C-β′	122.4	123.0	123.4	122.6
C-γ′	17.2	18.0	18.0	17.3
C-1′	130.9	130.8	131.0	131.4
C-2′	110.9	109.8	109.9	110.6
C-3′	143.4	149.9	150.1	143.3
C-4′	146.7	146.9	147.0	146.1
C-5′	133.3	115.7	116.2	133.2
C-6′	113.2	118.4	118.4	113.1
OCH_3	55.7	55.6	55.6	56.0

Table 4
^{13}C chemical shifts for carbons in **6**, **7a**, **7b** and **8** isolated from the reaction mixture of *Rhus* laccase-catalyzed oxidation of **5**. Solvent: DMSO-d_6

Carbons	^{13}C chemical shifts of dimeric products (δ)			
	6	**7a** (*threo*)	**7b** (*erythro*)	**8**
C-α	84.9	70.9	71.6	87.3
C-β	53.8	84.4	83.7	53.2
C-γ	71.0	60.2	60.2	63.2
C-1	132.2	133.3	133.1	132.3
C-2	110.4	111.4	111.2	110.6
C-3	147.5	147.0	147.0	147.1
C-4	145.9	145.5	145.5	146.3
C-5	115.1	114.5	114.6	114.8
C-6	118.6	119.5	119.0	118.4
OCH_3	55.6	55.6[a]	55.6[a]	55.6[a]
C-α′	84.9	128.7	128.7	129.4
C-β′	53.8	128.7	128.7	127.9
C-γ′	71.0	61.6	61.6	61.8
C-1′	132.2	130.0	130.1	130.4
C-2′	110.4	109.9	109.8	110.6
C-3′	147.5	149.8	149.8	143.5
C-4′	145.9	147.7	147.9	147.4
C-5′	115.1	115.6	115.6	128.9
C-6′	118.6	119.0	119.0	115.3
OCH_3	55.6	55.5	55.5	55.5

[a] These values are exchangeable.

4.6. Identification of dimeric and tetrameric products from the oxidation mixture of ***1***

2: mp 128–130°C (from EtOH), Lit. 130–131°C [14]. Si-gel *tlc*: R_f 0.54 (hexane/AcOEt, 3:2, v/v). EIMS (70 eV): *m/z* (rel. int.) 326 (M^+, 100). ^1H NMR (DMS-d_6): δ 1.30 (3H, *d*,

$J = 6.6$ Hz, γ-CH_3), 1.82 (3H, *d*, $J = 6.4$ Hz, γ′-CH_3), 3.40 (1H, *m*, H-β), 3.76 (3H, *s*, OCH_3) 3.78 (3H, *s*, OCH_3), 5.05 (1H, *d*, $J =$ 9.0 Hz, H-α), 6.14 (1H, *m*, H-β′), 6.34 (1H, *d*, $J = 15.7$ Hz, H-α′), 6.75–6.98 (5H, *m*, ArH).

3a: mp 119.8–124.5°C (from hexane–$CHCl_3$). Si-gel *tlc*: R_f 0.25 (hexane/AcOEt, 3:2, v/v). EIMS (70 eV): *m/z* (rel. int.) 344 (M^+, 11), 326 (12) 164 (100). ^{1}H NMR (DMS-d_6): δ 0.95 (3H, *d*, $J = 6.6$ Hz, γ-CH_3), 1.82 (3H, *d*, $J = 6.4$ Hz, γ′-CH_3), 3.75 (3H, *s*, OCH_3) 3.77 (3H, *s*, OCH_3), 4.40 (1H, *m*, H-β), 4.59 (1H, *t*, H-α), 5.17 (1H, *d*, $J = 4.2$ Hz, D_2O-exchangeable, α-OH) 6.15 (1H *m*, H-β′), 6.33 (1H, *d*, $J = 17.4$ Hz, H-α′), 6.69–7.00 (5H, *m*, ArH).

3b: Si-gel *tlc*: R_f 0.30 (hexane: AcOEt, 3:2, v/v). EIMS (70 eV): *m/z* (rel. int.) 344 (M^+, 11), 326 (7) 164 (100). ^{1}H NMR (DMS-d_6): δ 1.13 (3H, *d*, $J = 6.4$ Hz, γ-CH_3), 1.81 (3H, *d*, $J = 6.4$ Hz, γ′-CH_3), 3.74 (3H, *s*, OCH_3) 3.78 (3H, *s*, OCH_3), 4.43 (1H, *m*, H-β), 4.59 (1H, *t*, H-α), 5.19 (1H, *d*, $J = 4.6$ Hz, D_2-exchangeable, α-OH) 6.12 (1H *m*, H-β′), 6.31 (1H, *d*, $J = 17.4$ Hz, H-α′), 6.67–6.97 (5H, *m*, ArH).

Dehydrodi(dehydrodiisoeugenol) (**4**) : mp 140–145°C (from hexane–$CHCl_3$). Si-gel *tlc*: R_f 0.18 (hexane: AcOEt, 3:2, v/v). EIMS (70 eV): *m/z* (rel. int.) 650 (M^+, 100). ^{1}H NMR (DMS-d_6): δ 1.32 (6H, *d*, $J = 6.8$ Hz, γ-CH_3), 1.82 (6H, *d*, $J = 6.4$ Hz, γ′-CH_3), 3.43 (2H, *m*, H-β), 3.77 (6H, *s*, OCH_3) 3.82 (6H, *s*, OCH_3), 5.10 (2H, *d*, $J = 9.0$ Hz, H-α), 6.13 (2H *m*, H-β′), 6.35 (2H, *d*, $J = 15.9$ Hz, H-α′), 6.80–6.99 (8H, *m*, ArH).

*4.7. Identification of dimeric products from the oxidation mixture of **5***

6: mp 153–155°C (from EtOH); Lit. 156–157°C [16]. Si-gel *tlc*: R_f 0.41 ($CHCl_3$: MeOH, 9:1, v/v). EIMS (70 eV): *m/z* (rel. int.) 358 (M^+, 95), 340 (100). ^{1}H NMR (DMS-d_6): δ 3.43 (1H, *dd*, H-β), 3.75 (2H, *d*, γ-CH_2), 3.73 (6H, *s*, OCH_3), 4.09 (2H, *d*, $J = 6.2$ Hz, γ′-CH_2), 5.46 (1H, *d*, $J = 9.6$ Hz, H-α), 6.19 (1H *dt*, $J_{\alpha'\beta'} = 15.9$, $J_{\beta'\gamma'} = 6.2$ Hz, H-β′), 6.46 (1H, *d*, $J_{\alpha'\beta'} = 15.9$ Hz, H-α′), 6.72–6.97 (5H, *m*, ArH).

7a: Si-gel *tlc*: R_f 0.38 ($CHCl_3$: MeOH, 9:1, v/v). Colorless resinous material; it did not be crystallized but consisted of almost single substance as shown by GC and *tlc*. EIMS (70 eV): *m/z* (rel. int.) 376 (M^+, 27), 206 (100). ^{1}H NMR (DMS-d_6): δ 3.03 (2H, *q*, H-β) 3.59 (2H, *m*, γ-CH_2OH) 3.73 (6H, *s*, OCH_3) 4.09 (2H, *m*, γ′-CH_2OH) 4.26 (1H, *m*, H-β), 4.62 (1H, *t*, D_2O-exchangeable, γ-CH_2OH) 4.72 (1H, *t*, H-α), 4.79 (1H, *t*, D_2O-exchangeable, γ′-CH_2OH) 5.25 (1H, *d*, D_2O-exchangeable, α-OH) 6.23 (1H, *m*, H-β′) 6.45 (1H *d*, H-α′), 6.61–7.07 (5H, *m*, ArH).

8: mp 107–110°C (from EtOH); Lit. 110–111°C [17]. Si-gel *tlc*: R_f 0.57 ($CHCl_3$: MeOH, 9:1, v/v). EIMS (70 eV): *m/z* (rel. int.) 358 (M^+, 34), 137 (100). ^{1}H NMR (DMS-d_6): δ 303(2H, *m*, H-β), 3.73 (2H, *dd*, γ-CH_AH_B) 3.77 (6H, *s*, OCH_3), 4.12 (2H, *dd*, γ-CH_AH_B), 4.61 (2H, *d*, $J = 4.7$ Hz, H-α), 6.73–6.92 (6H, *m*, ArH).

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