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Effect of pH on the Dehydrogenative Polymerization of Monolignols by Laccases from *Trametes versicolor* and *Rhus vernicifera*

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Cite This: *ACS Omega* 2022, 7, 9846−9852

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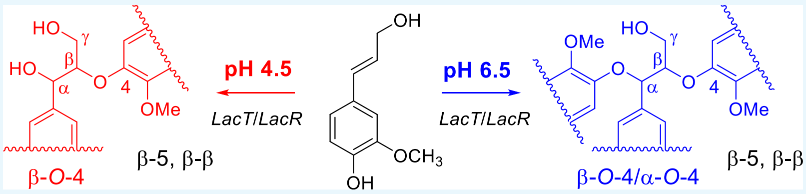

ABSTRACT: Dehydrogenative polymerization of coniferyl alcohol (CA) and sinapyl alcohol (SA) was conducted using commercial laccases, fungal laccase from *Trametes versicolor* (*LacT*) and plant laccase from *Rhus vernicifera* (*LacR*), at pH 4−7 to investigate how the enzymatic polymerization of monolignols differs between these two laccase systems. The enzyme activity of *LacT* was the highest at pH 4, whereas that of *LacR* was the highest at pH 7. A dehydrogenation polymer (DHP) was obtained only from CA in both laccase systems, although the consumption rate of SA was higher than that of CA. $^{1}H-^{13}C$ HSQC NMR analysis showed that DHPs obtained using *LacT* and *LacR* contained lignin substructures, including *β*-*O*-4, *β*-*O*-4/*α*-*O*-4, *β*-*β*, and *β*-5 structures. At pH 4.5, the *β*-*O*-4 structure was preferentially formed over the *β*-*O*-4/*α*-*O*-4 structure, whereas at pH 6.5, the *β*-*O*-4/*α*-*O*-4 structure was preferred. The pH of the reaction solution was more vital to affect the chemical structure of DHP than the origin of laccases.

■ INTRODUCTION

Lignin is one of the most abundant biopolymers found in nature. The lignin structure is quite complicated and comprises many substructures, including *β*-*O*-4, *β*-5, *β*-*β*, 5-5, and 4-*O*-5 structures as shown in Figure 1A.[1] These substructures are formed from the enzymatic dehydrogenative polymerization of monolignols, including coniferyl alcohol (CA), sinapyl alcohol (SA), and *p*-coumaryl alcohol (Figure 1B). Nontraditional monolignols are also reported to be involved in lignin biosynthesis.[2] These monolignols are oxidized by peroxidases and/or laccases, and the resulting monolignol radicals couple with each other to produce lignin dimers. Under endwise polymerization, the dimers are further oxidized and coupled with monolignol radicals to form lignin trimers. Further oxidation of the phenolic end group of the growing polymer and coupling with monolignol radicals are repeated to produce lignin.[1−3]

To investigate the structure of natural lignin, synthetic lignin derived from the dehydrogenative polymerization of monolignols has long been used.[4] In previous investigations, dehydrogenative polymerization of CA and SA was conducted using horseradish peroxidase (HRP), and the presence of both guaiacyl and syringyl units increased the *β*-*O*-4 structures in natural and synthetic lignins.[5] Silver(I) oxide was also used to investigate the radical coupling of monolignols. Monolignol acylates, including coniferyl acetate, sinapyl acetate, and sinapyl *p*-coumarate were used, and the effects of acyl groups on the lignin substructures were studied.[6,7]

Recent investigations on lignin biosynthesis suggest that peroxidase/H_2O_2 and laccase/O_2 play an essential role in lignin polymerization in many plant species.[8,9] Especially, laccase draws increasing attention, and the vital role of laccases in the lignification was shown through the knockout mutants in Arabidopsis.[10−12] However, studies on synthetic lignins obtained from the dehydrogenative polymerization of monolignols using laccases have drawn less attention. Only a few studies were reported on the structural analysis of synthetic lignins obtained using laccases.[13−16]

Laccase is a multicopper oxidase with a high degree of structural conservation among bacteria, fungi, and plants.[17] Basic enzyme activities of laccases are well elucidated using various substrates, including hydroquinone, guaiacol, catechol, caffeic acid, 2,6-dimethoxyphenol, and syringaldazine.[17,18] The optimal pH of laccase activity for the phenolic substrate depends on the origin of laccases.[17] The optimal pH for phenolic substrates is 3−5 for most fungal laccases, including *Trametes versicolor*. For plant laccases, the optimal pH range is 5−7.

Received: January 8, 2022
Accepted: February 24, 2022
Published: March 7, 2022

https://doi.org/10.1021/acsomega.2c00144
ACS Omega 2022, 7, 9846−9852

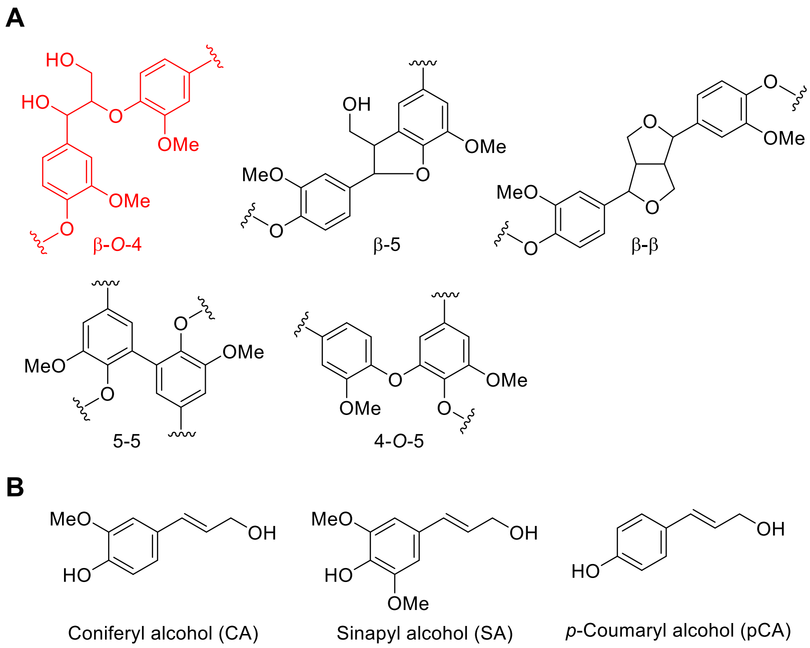

Figure 1. Chemical structures. (A) Substructures in softwood lignin. The most abundant *β-O*-4 structure is highlighted in red. (B) Monolignols.

In this study, the dehydrogenative polymerization of CA and SA was performed using commercial laccases, fungal laccase from *T. versicolor* (*LacT*) and plant laccase from *Rhus vernicifera* (*LacR*), to study how the dehydrogenative polymerization varies between these laccase systems. The enzyme activities of laccases were compared at different pHs. In particular, the effect of pH on the structure of the obtained dehydrogenation polymer (DHP) was analyzed using $^{1}H-^{13}C$ correlation heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectroscopy.

■ MATERIALS AND METHODS

General. All chemicals were bought from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) or Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). CA and SA were synthesized from ethyl ferulate and ethyl sinapate, respectively.[5] NMR spectra were recorded using a Bruker AVANCE II 500 FT-NMR (500 MHz) spectrometer or a Bruker AVANCE Neo 500 FT-NMR (500 MHz) in DMSO-d_6 or acetone-d_6. The central peaks of the residual dimethyl sulfoxide (DMSO) (^{1}H: 2.50 ppm, ^{13}C: 39.52 ppm) and acetone (^{1}H: 2.05 ppm, ^{13}C: 29.84 ppm) were used as internal references.

Laccases. *LacT* and *LacR* were bought from Sigma-Aldrich Japan (Tokyo, Japan). The molecular mass of *LacT* and *LacR* was estimated to be 54.7 and 82.9 kDa, respectively, using sodium dodecyl sulfate−polyacrylamide gel electrophoresis. *LacT* was used without purification. *LacR* from lacquer tree was purified before use because it contained resinous materials from lacquer. *LacR* (30 mg) was suspended in acetone (1.0 mL) and ground using a spatula and an ultrasonic bath for 3 min. The supernatant acetone solution was transferred to a 15 mL tube, and the residue was further ground in water (1.0 mL). The acetone solution and the residue in water were combined and lyophilized. The ground dry powder was kept in a freezer. For the dehydrogenation reactions, the ground powder was suspended in water using a vortex mixer and centrifuged for 1 min (12,300 *g*), and the supernatant solution was used.

The protein content of laccase was measured using the UV method at 280 nm and calibrated with bovine serum albumin as a standard (Quick Start BSA Standard Set, Bio-Rad, Hercules, CA, USA).

Relative Enzyme Activity of Laccases. Relative enzyme activities of laccases were determined using 2,6-dimethoxyphenol as a substrate. A 2,6-dimethoxyphenol solution (0.1 mol/L, 500 μL), a buffer solution (50 mmol/L, pH 4−7, 500 μL), and an aqueous laccase solution (*LacT*: 0.05 mg/mL, 500 μL; *LacR*: 0.25 mg/mL, 500 μL) were mixed, and the increase in absorbance was measured at 470 nm at 30 °C for 10 min using a spectrophotometer (Shimazu UV-1800, Kyoto, Japan). One unit of the enzyme was defined as the amount that produced 1 μmol of the oxidized product (coerulignone, 49.6 mM^{-1} cm^{-1} at 470 nm) in 1 min.

Dehydrogenative Polymerization of Monolignol by Laccase. In a typical procedure, to a stirred solution of CA (18.0 mg, 0.1 mmol) or SA (21.0 mg, 0.1 mmol) in 9 mL of a buffer solution (pH 4−7) in a round bottom flask (25 mL), 1 mL of laccase solution (0.5 mg/mL for *LacT* or 3 mg/mL for *LacR*) was added at 30 °C ("Zulauf" method). At a prescribed time, an aliquot (250 μL) of the reaction mixture was withdrawn, and ethyl acetate (500 μL), aq 3,4-dimethoxyacetophenone solution (1.8 mg/mL, 250 μL, internal standard), and 0.1 mol/L HCl solution (250 μL) were added to the mixture. The mixture was shaken, and ethyl acetate solution was dried over Na_2SO_4 and concentrated to dryness in vacuo. The reaction products were diluted using methanol (500 μL) and analyzed using high-performance liquid chromatography (HPLC) on a Gilson liquid chromatography system (WI, USA) with a UV/vis detector model 118 (280 nm). A YMC-Triart Phenyl column (15 cm × 4.6 mm) (Kyoto, Japan) was used at 30 °C. The solvent system was a gradient of methanol (A) and 0.1% formic acid (B) with a flow rate of 1.0 ml/min. The gradient was as follows: 25% A for 30 min, and from 25 to 50% A in 25 min. The retention times of CA, SA, and 3,4-dimethoxy phenol were 12.4, 13.3, and 36.5 min, respectively.

After the completion of the reaction, the reaction mixtures were centrifuged at 3000 rpm (approx. 1500*g*) for 15 min. The precipitates were washed using water, lyophilized, and further dried in vacuo over P_2O_5 to give DHP. The supernatant solution was extracted using ethyl acetate (10 mL × 3). The ethyl acetate solution was washed with brine, dried over Na_2SO_4, and concentrated to dryness in vacuo to give ethyl acetate extracts that were unprecipitated.

Size Exclusion Chromatography of Dehydrogenative Polymerization Products. The obtained DHP was acetylated with acetic anhydride and pyridine and analyzed using size exclusion chromatography. The sample solution was filtered and injected into Shodex GPC packed columns GPC KF-802 + KF-803L × 2 (30 cm × 8 mm) using a JASCO liquid chromatography system equipped with a UV/vis detector UV-975 (Tokyo, Japan). Tetrahydrofuran was used as an eluent with a flow rate of 1.0 mL/min at 40 °C. Polystyrene standards PStQuick E and F (Tosoh, Tokyo, Japan) were used for molecular mass calibration.

■ RESULTS AND DISCUSSION

Enzyme Activities of Laccases from *T. versicolor* and *R. vernicifera* at Different pHs. The enzyme activities of *LacT* and *LacR* were measured in sodium acetate buffer (pH 4−4.5) and potassium phosphate buffer (pH 4−7) using 2,6-dimethoxyphenol as a substrate. *LacT* was used without purification. *LacR* was purified before use. The protein contents in *LacT* and purified *LacR* were 17.9 and 27.7%, respectively. The relative activity of *LacT* is indicated in Figure 2A. The maximum enzyme activity of *LacT* was 156 unit/g (100%) at pH 4.0 in acetate buffer. In a phosphate buffer, the activity was a little lower than that in acetate buffer. The enzyme activity of *LacT* for the oxidation of 2,6-dimethoxyphenol reduced with the increase in the pH, and it was almost lost at pH 7.0 in the phosphate buffer. Most fungal laccases have an optimum pH range of 3.0−5.0 for phenolic substrates.[17,19]

In contrast, the enzyme activity of *LacR* was the highest at pH 7.0 (10.7 unit/g, 100%) in the phosphate buffer, as shown in Figure 2B. The activity reduced with a reduction in pH, and it was only 1.1 unit/g at pH 4.0 in the phosphate buffer. In the acetate buffer, the enzyme activity of *LacR* was significantly higher than that in the phosphate buffer. The high enzyme activity under neutral conditions for *LacR* using 2,6-dimethoxyphenol as a substrate was consistent with the reported observation using isoeugenol and CA as substrates.[15] Plant laccases have their optimum pH range nearer to the physiological range.[17] The enzyme activity of *LacR* was much lower than that of *LacT*, and the optimum pH range differed from each other. The basic enzyme activities of *LacR* and *LacT* were quite different from each other.

Dehydrogenative Polymerization of CA and SA by Laccases from *T. versicolor* and *R. vernicifera*. The phosphate buffer was used for the dehydrogenative polymerization of monolignols because acetic acid was introduced into the collected DHP molecules in preliminary experiments when acetate buffer was used. Monolignols, CA, and SA were treated separately with *LacT* and *LacR* at pH 4.5 and 6.5 in phosphate buffer. An aliquot of the reaction mixture was withdrawn at a prescribed time, and residual monolignols were analyzed by HPLC. The reactivity of monolignols using *LacT* is indicated in Figure 3A. The enzyme activity of *LacT* for CA and SA at pH 4.5 was higher than that at pH 6.5, which was the same as that when 2,6-dimethoxyphenol was used as a substrate. For CA and SA, almost all monolignols were consumed within 4−5 h at pH 4.5, whereas at pH 6.5, more than 60% of monolignols remained unreacted after 6 h reactions. The reactivity of SA toward *LacT* was slightly higher than that of CA at pH 4.5 and 6.5.

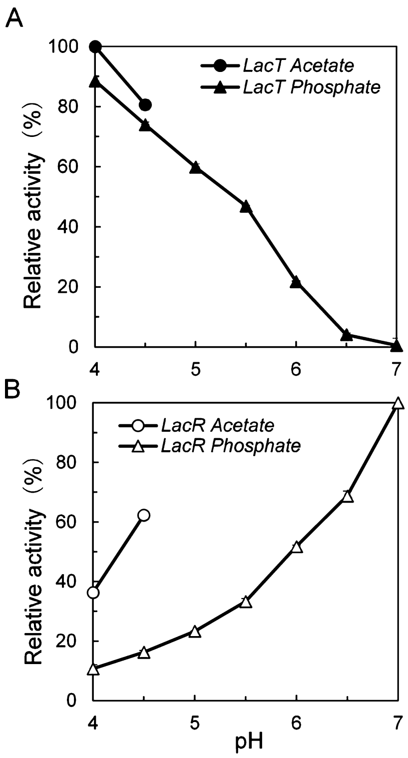

Figure 2. Effect of pH on the enzyme activity using 2,6-dimethoxyphenol as a substrate. (A) Relative activity of *LacT*. Solid circle: acetate buffer, solid triangle: phosphate buffer. The maximum activity was 156 unit/g (100%) at pH 4 in sodium acetate buffer. (B) Relative activity of *LacR*. Open circle: acetate buffer and open triangle: phosphate buffer. The maximum activity was 10.7 unit/g (100%) at pH 7 in potassium phosphate buffer.

Figure 3B shows the reactivity of monolignols by *LacR* at pH 4.5 and 6.5. The enzyme activity of *LacR* for CA and SA at pH 6.5 was higher than that at pH 4.5. The difference in the reactivity of monolignols between pH 4.5 and pH 6.5 was more evident when SA was used as a substrate. SA was completely consumed by *LacR* at pH 6.5 in 4−6 h, whereas CA needed more than 10 h to complete the reactions at pH 4.5 and pH 6.5. The higher reactivity of SA than CA was observed both at pH 4.5 and pH 6.5, which was the same as when *LacT* was used. The higher enzyme activity for SA than CA was reported for maple laccase and *Rhus* laccase at neutral pH.[13,16]

Table 1 summarizes the yields of DHP and ethyl acetate extracts from the dehydrogenative polymerization of CA and SA by *LacT* and *LacR*. After monolignols were completely consumed, reaction mixtures were centrifuged, and precipitates were obtained and dried. The obtained precipitate was referred to as DHP. The yield of DHP from dehydrogenative polymerization of CA by *LacT* at pH 4.5 was 68 wt %, which was higher than that at pH 6.5. In contrast, SA did not form DHP by *LacT* at any pH, although the consumption of SA by *LacT* was faster than that of CA. The reaction products from SA by *LacT* were recovered as ethyl acetate extracts.

https://doi.org/10.1021/acsomega.2c00144
ACS Omega 2022, 7, 9846−9852

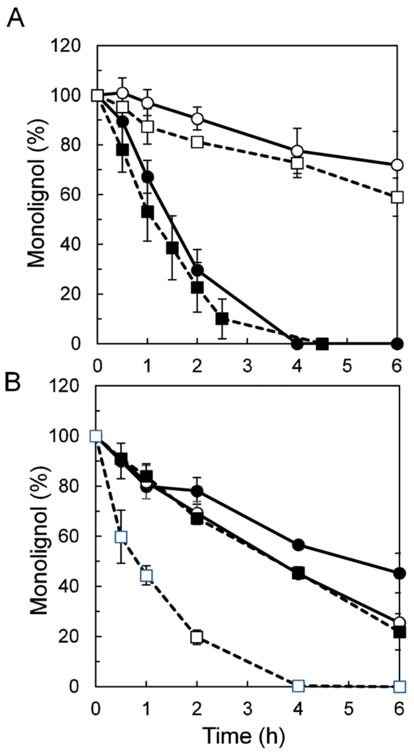

Figure 3. Reactivity of coniferyl alcohol (CA) and sinapyl alcohol (SA) at pH 4.5 and 6.5 in phosphate buffer using (A) *LacT* and (B) *LacR*. Solid circle: CA at pH 4.5, open circle: CA at pH 6.5, solid square: SA at pH 4.5, and open square: SA at pH 6.5.

Table 1. Yields of DHP and Ethyl Acetate Extracts from Dehydrogenative Polymerization of CA and SA by *LacT* and *LacR*

laccase	substrate	pH	reaction time[a] (h)	products[b]	yield (wt %)
LacT	CA	4.5	6	EtOAc	36.5 ± 15.5
				DHP	68.0 ± 19.3
LacT	CA	6.5	48	EtOAc	82.3 ± 13.8
				DHP	24.1 ± 0.9
LacT	SA	4.5	4.5	EtOAc	103 ± 5.2
				DHP	0
LacT	SA	6.5	28	EtOAc	104 ± 13.3
				DHP	0
LacR	CA	4.5	30	EtOAc	82.4 ± 5.0
				DHP	trace
LacR	CA	6.5	10	EtOAc	67.2 ± 19.1
				DHP	59.6 ± 24.4
LacR	SA	4.5	12	EtOAc	102 ± 5.5
				DHP	0
LacR	SA	6.5	6	EtOAc	79.6 ± 22.5
				DHP	0[c]

[a]Reaction time: the time required for all monolignols to be consumed. [b]EtOAc: ethyl acetate extracts. [c]Syringaresinol (*β*-*β* dimer, 25 wt %) was obtained as precipitates.

After the 10 h reaction of CA by *LacR* at pH 6.5, 60 wt % of DHP was collected, whereas only a trace amount of DHP was derived from CA by *LacR* at pH 4.5. Alternatively, SA did not form DHP at all using *LacR* at pH 4.5. When SA was treated using *LacR* at pH 6.5, a significant amount of precipitate (25 wt %) was obtained. However, the precipitate was found to be a *β*−*β* type dimer (syringaresinol) by NMR analysis. Thus, this dimer compound was not considered DHP. These results indicate that *LacR* cannot produce DHP from SA at neutral or acidic pHs. It was also reported that DHP from SA was hardly produced by *Rhus* laccase at pH 6.5 by the Zutropf method (gradual addition of monolignol: end-wise polymerization).[16] From our experimental data and the reported results, it can be concluded that either fungal laccase *LacT* or plant laccase *LacR* cannot produce DHP from SA at acidic or neutral pHs.

Structural Analysis of DHP from CA by Laccases from *T. versicolor* and *R. vernicifera*. Table 2 shows the molecular mass of the DHPs obtained from dehydrogenative polymerization (Zulauf method: bulk polymerization) of CA by *LacT* at pH 4.5 and 6.5 and using *LacR* at pH 6.5. Weight average molecular mass (M_w) of the DHP from CA by *LacT* at pH 4.5 was 1197, corresponding to a tetramer or pentamer. M_w of DHPs from CA by *LacT* at pH 6.5 and by *LacR* at pH 6.5 was similar and corresponds to a trimer. Polydispersities of DHPs were small, and they are identical to each other (M_w/M_n = 1.2−1.3).

Table 2. Molecular Mass of DHPs Obtained by the Dehydrogenative Polymerization of CA by Laccases

entry	laccase	pH	M_n	M_w	M_w/M_n
1	*LacT*	4.5	914 ± 55	1197 ± 90	1.31 ± 0
2	*LacT*	6.5	715 ± 33	867 ± 43	1.21 ± 0
3	*LacR*	6.5	679	853	1.26

The molecular mass of the DHP obtained from CA by *LacT* and *LacR* in this investigation was small compared with those obtained using HRP in our previous investigations.[5] These experiments using *LacT* and *LacR* were conducted using the Zulauf method (addition of monolignol at once), whereas the previous HRP experiments were conducted using the Zutropf method. The gradual addition of monolignol (Zutropf method) leads to the end-wise polymerization, which makes the molecular mass of DHP higher. The low molecular mass of the DHP in this investigation is partly due to the Zulauf method. Further experiments are necessary to clarify the effect of laccase on the molecular mass of DHP.

Figure 4 shows the HSQC NMR spectra of DHPs from dehydrogenative polymerization of CA using *LacT* and *LacR* in DMSO-d_6. HSQC NMR analysis can provide detailed information on the lignin substructures in the DHP obtained using *LacT* and *LacR*. Lignin substructures *β*-*O*-4 (A), *β*-5 (B), and *β*-*β* (C) were seen in all DHP samples (Figure 4). The basic structure of DHPs obtained using *LacT* and *LacR* was similar to those obtained by enzymatic polymerization of CA using HRP. In addition to the primary lignin substructures, a minor lignin substructure, *β*-*O*-4/*α*-*O*-4 (A′), was observed. The *β*-*O*-4/*α*-*O*-4 (A′) structure has not often been reported in softwood or hardwood lignins.[20,21] The *β*-*O*-4/*α*-*O*-4 (A′) structure was undetected in milled wood lignin isolated either from todo fir or white birch in our investigations.[22,23] In contrast, the *β*-*O*-4/*α*-*O*-4 (A′) structure was observed in mature and immature bamboo lignins in our previous investigations.[24,25] Bamboo-cultured cell lignin also contains the *β*-*O*-4/*α*-*O*-4 (A′) structure.[26]

Table 3 shows the frequency of the lignin substructures in DHPs, which was estimated by regarding *β*-*O*-4 (A), *β*-*O*-4/*α*-*O*-4 (A′), *β*-5 (B), and *β*-*β* (C) structures totally as 100% of the side chain structures. The signal intensities of *β*-*O*-4 (A*α*), *β*-*O*-4/*α*-*O*-4 (A′*α*), *β*-5 (B*α*), and *β*-*β* (C*α*) were used for the

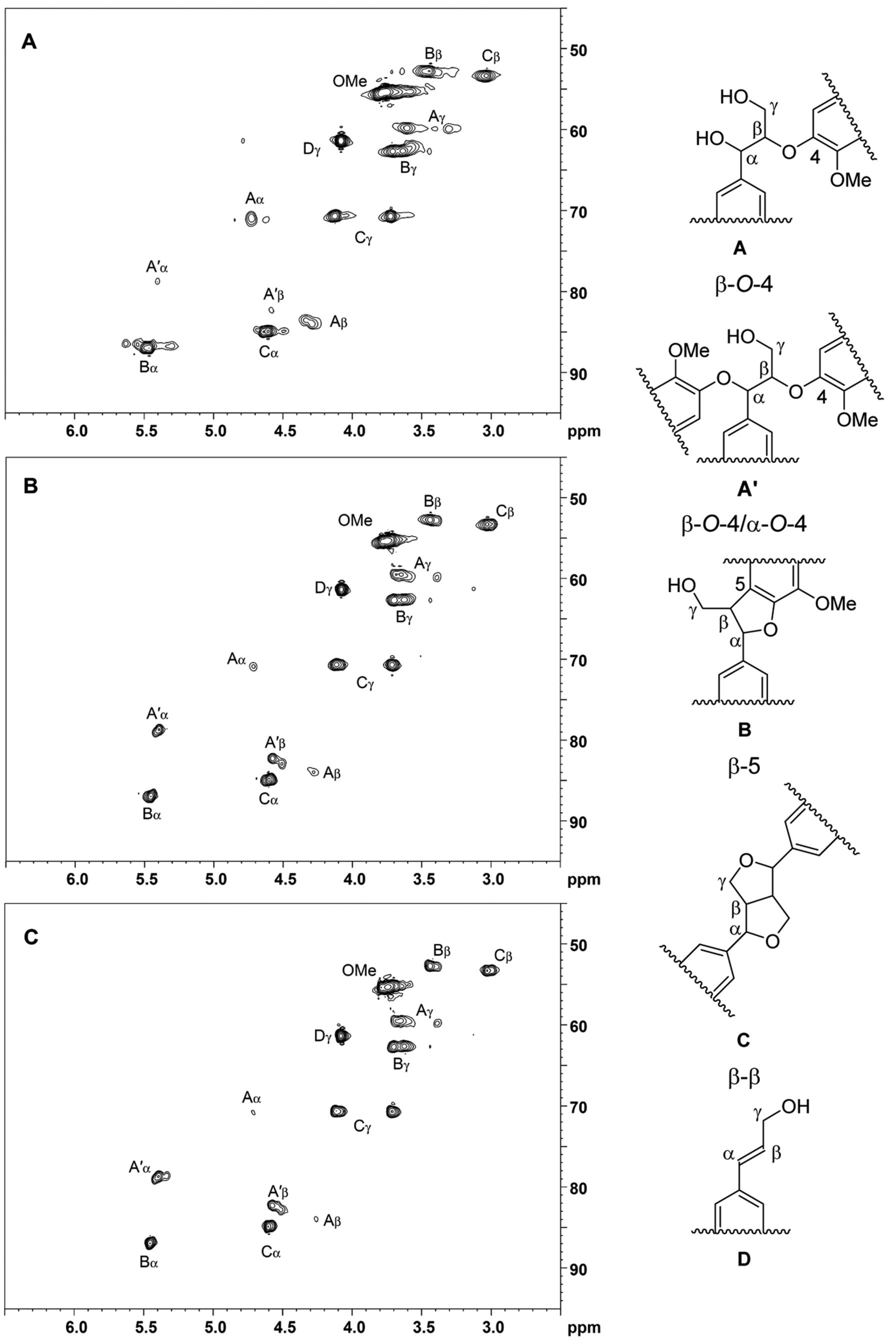

Figure 4. HSQC NMR spectra of DHPs from dehydrogenative polymerization of CA by (A) *LacT* at pH4.5, (B) *LacT* at pH6.5, and (C) *LacR* at pH6.5 in DMSO-d_6.

Table 3. Relative Abundance of Substructures in DHPs Estimated by HSQC NMR Analysis

entry	laccase	pH	*β*-O-4	*β*-O-4/*α*-O-4	*β*-5	*β*-*β*
1	*LacT*	4.5	11.7 ± 0	1.1 ± 0.2	56.8 ± 1.0	30.5 ± 1.2
2[a]	*LacR*	4.5	20.1 ± 0.2	0.2 ± 0.2	59.3 ± 0.1	20.5 ± 0.5
3	*LacT*	6.5	3.4 ± 0.3	18.8 ± 0.8	47.0 ± 0.6	30.8 ± 0.1
4	*LacR*	6.5	1.9 ± 0.2	26.4 ± 0.4	44.6 ± 1.6	27.1 ± 1.0

[a]Ethyl acetate extracts were analyzed because DHP (precipitates) was not obtained.

analysis. For *LacR* at pH 4.5, ethyl acetate extracts were assessed instead of DHP because only a trace amount of DHP was obtained under the conditions used.

The most abundant substructure in DHP obtained using *LacT* at pH 4.5 (Zularf method) was the *β*-5 (B) structure (56.8%), followed by the *β*-*β* (C) (30.5%) and the *β*-*O*-4 (A) structures (11.7%). A small amount of the *β*-*O*-4/*α*-*O*-4 (A′) structure (1.1%) was also observed at pH 4.5. Similarly, in ethyl acetate extracts collected using *LacR* at pH 4.5, the frequency of the *β*-5 (B) structure (59.3%) was the highest,

https://doi.org/10.1021/acsomega.2c00144
ACS Omega 2022, 7, 9846−9852

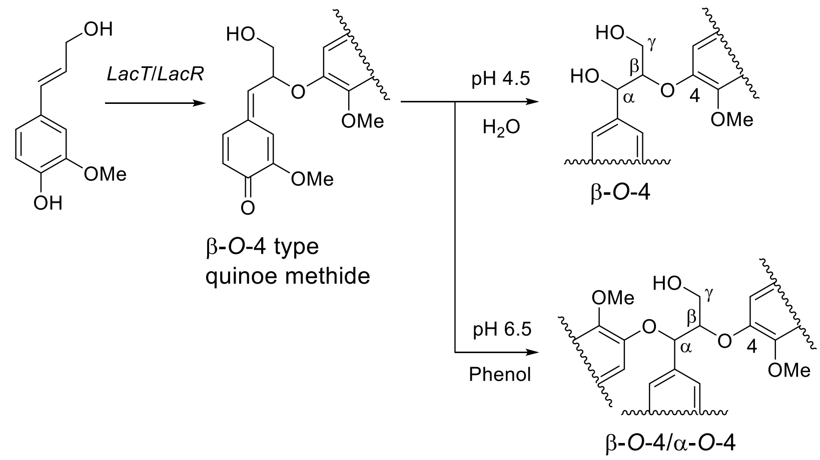

Figure 5. Reaction of *β*-*O*-4 type quinone methide under different pH conditions during dehydrogenative polymerization of CA using *LacT* and *LacR*.

followed by the *β*-*β* (C) (20.5%) and the *β*-*O*-4 (A) structures (20.1%). The frequency of the *β*-*O*-4/*α*-*O*-4 (A′) structure (0.2%) was low.

Alternatively, in the case of *LacT* at pH 6.5, the proportion of the *β*-5 (B) was the highest (47.0%), followed by the *β*-*β* (C) (30.8%) structures. The amount of the *β*-*O*-4/*α*-*O*-4 (A′) structure became more significant, and the relative proportion of the *β*-*O*-4/*α*-*O*-4 (A′) structure reached 18.8%. The frequency of the *β*-*O*-4 (A) structure was only 3.4%. A high proportion of the *β*-*O*-4/*α*-*O*-4 structure (26.4%) was also observed using *LacR* at pH6.5. In the case of *LacR* at pH 6.5, the proportions of *β*-5 (B), *β*-*β* (C), *β*-*O*-4/*α*-*O*-4 (A′), and *β*-*O*-4 (A) structures were 46.6, 27.1, 26.4, and 1.9%, respectively. The proportions by *LacR* at pH 6.5 were similar to those obtained using *LacT* at pH 6.5. These results showed that the reaction pH was more critical for affecting the chemical structure of dehydrogenative polymerization products than the origin of laccase.

The dehydrogenative polymerization of CA using *Rhus* laccases under neutral conditions has been reported. Okusa et al. reported that *Rhus* laccase oxidized CA in acetone-water very slowly, and 20% of *β*−*β* dimer, 25% of *β*-5 dimer, and 2% of *β*-*O*-4 dimer were obtained at a reaction time of 144 h.[14] Matsumoto et al. reported that DHP derived from CA contained the *β*-5 and *β*-*β* structures, but not the *β*-*O*-4 structure using *Rhus* laccase at pH 6.5 (Zutropf method).[16] The reported high proportions of *β*-5 and *β*-*β* structures under neutral conditions were consistent with our results. However, the most significant differences from their results were that we observed a high proportion of the *β*-*O*-4/*α*-*O*-4 structure at pH 6.5 and a high proportion of the *β*-*O*-4 structure at pH 4.5 for both *LacT* and *LacR*. These results can be well explained by the reaction of *β*-*O*-4 type quinone methide as shown in Figure 5. It is reported that in the quinone methide reactions with vanillyl alcohol in aqueous solution, only the addition of water was observed at low pH, and the phenol addition was prominent under neutral conditions.[27] A similar pH effect has been reported for the dehydrogenative polymerization of SA using HRP.[28] These results suggest that in the biosynthesis of lignin, the type of laccase does not significantly affect the structure of lignin. It is likely that the surrounding environment during lignin biosynthesis, such as pH, has a greater influence on the structure of lignin. Because the high proportion of the *β*-*O*-4/*α*-*O*-4 structure is not observed for native lignins,[20−23] lower pH is most likely a lignin biosynthesis condition as suggested by some studies on the reactivity of quinone methide[29,30] and on the dehydrogenative polymerization of monolignol using HRP.[28]

■ CONCLUSIONS

The fungal laccase *LacT* and plant laccase *LacR* oxidized CA and SA at pH 4.5 and 6.5, respectively. However, DHP was obtained only from CA. Main lignin substructures, including *β*-*O*-4, *β*-*O*-4/*α*-*O*-4, *β*-*β*, and *β*-5 structures, were observed in all DHPs. At pH 6.5, the *β*-*O*-4/*α*-*O*-4 (A′) structure was preferentially formed over the *β*-*O*-4 (A) structure for both *LacT* and *LacR*, which is different from the actual biosynthesis of lignin. In contrast, dehydrogenative polymerization products obtained at pH 4.5 by *LacT* and *LacR* had a much higher proportion of the *β*-*O*-4 (A) structure than the *β*-*O*-4/*α*-*O*-4 (A′) structure. The pH of the reaction solution had a greater effect on the structure of the resulting dehydrogenative polymerization products than the origin of laccase. These findings will contribute to our further understanding of the structure and biosynthesis of lignin.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Part of this work was supported by JSPS KAKENHI grant number 20K06169.

■ ABBREVIATIONS

CA, coniferyl alcohol; DHP, dehydrogenation polymer; HSQC, heteronuclear single quantum coherence; LacT, laccase from *Trametes versicolor*; LacR, laccase from *Rhus vernicifera*; NMR, nuclear magnetic resonance; SA, sinapyl alcohol

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https://doi.org/10.1021/acsomega.2c00144
ACS Omega 2022, 7, 9846−9852