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Comparison of Dehydrogenation Polymers by Commercial Enzymes, Laccase from *Rhus vernicifera* and Horseradish Peroxidase

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Abstract
Dehydrogenation polymers (DHPs) were prepared by laccase from *Rhus vernicifera* and horseradish peroxidase (HRP). The enzymatic ability of oxidation and polymerization was compared between these enzymes. Laccase showed higher enzyme activity against syringaldazine than ABTS, while HRP exhibited lower enzyme activity against syringaldazine than 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). These enzymes had various enzyme activity against these substrates. DHPs from sinapyl alcohol (SA) were hardly produced by each enzyme, whereas DHPs from coniferyl alcohol (CA) were produced by both enzymes. The laccase oxidized sinapyl alcohol faster than coniferyl alcohol. The nuclear magnetic resonance (NMR) analysis demonstrated that acetylated-DHP (Ac-DHP) from CA by laccase contained β-5 and β-β linkages, but not β-*O*-4 linkage. On the other hand, Ac-DHP from CA by HRP carried all these three linkages.
***Keywords**: Dehydrogenation polymers (DHPs); Laccase; Lignin*

INTRODUCTION

Lignin is a natural aromatic polymer, with much higher carbon content than other major wood components such as cellulose and hemicellulose. Lignin is expected to serve as a new carbon resource alternative to fossil resources [1)]. Many studies have been trying to use lignin as industrial raw material [2,3)]. However, there are still unexplained problems as to its structure and formation process [4)]. These problems hamper industrial lignin application. The final step in lignin formation is initiated by enzymatic oxidation (dehydrogenation) [5)]. The enzymes oxidize lignin precursors such as coniferyl alcohol (CA) and sinapyl alcohol (SA), and radical coupling consequently occurs. Dehydrogenative polymer (DHP) is prepared to mimic these biological processes *in vitro* and typically used as a lignin model compound [6)]. In many studies, horseradish peroxidase (HRP) has been used to prepare DHP as a monolignol-dehydrogenation enzyme and the resulting DHP has been analyzed in terms of the frequency of its interunitary linkages, molecular mass and other physical & chemical properties [7-10)]. It has often been reported that by using HRP, DHP can be successfully obtained from CA, but not from SA [11,12)].

It is generally considered that not only peroxidase but also laccase works as a monolignol-dehydrogenation enzyme that initiates lignin polymerization [13)]. Peroxidases catalyze the oxidation of a substrate, using hydrogen peroxide. In particular, HRP has a heme in its catalytic center [14)]. On the other hand, laccases are multicopper oxidases and catalyze oxidation with a reduction of oxygen to water [15,16)]. Thus, these two enzymes have quite different oxidation systems. This difference can also affect how DHP forms, depending on what types of enzyme (HRP or laccase) are used. However, while DHP formation *via* HRP oxidation has been studied extensively, DHP formation *via* laccase oxidation has not so far. One of the major reasons is that isolation of laccase from wood without losing its activity is quite difficult. But there are a few exceptional studies: some laccases were used to prepare DHP [17-23)]. In the case of Sycamore Maple laccase, DHP was successfully obtained from CA. but not from SA [17,18)]. Loblolly Pine laccase was found to oxidize both CA and SA to form DHP oligomers, if not polymers [19)].

There are some discrepancies among these studies on a wood laccase. Higuchi reported that laccase from *Rhus vernicifera* oxidized CA and the resulting DHP was produced [20], while Nakamura showed laccase from *R. vernicifera* did not oxidize CA [21]. Okusa *et al*. said laccase from *R. vernicifera* oxidized CA very slowly [22]. Shiba *et al*. reported laccase from *R. vernicifera* oxidized CA, but gave no resulting DHP [23]. Thus, DHPs by the use of some laccases were prepared and the functions of these enzymes were evaluated, especially in terms of whether they can oxidize CA, SA or both, and/or produce DHP. However, the number of published studies on laccase-catalyzed DHP formation using both CA and SA is quite limited. Practically no research to clarify the occurrence and the frequency of the interunitary linkages of the laccase-catalyzed DHPs has been published, as far as we know.

In this study, we prepared DHPs or DHP oligomers from CA and SA, using laccase from *R. vernicifera* and HRP, focusing on evaluating the ability of the enzymes to oxidize and/or polymerize these monolignols during DHP formation process. The interunitary linkages of the DHPs were also comparatively analyzed by 2D NMR (Heteronuclear Single Quantum Correlation, HSQC) technique.

EXPERIMENTAL

Materials and Methods

Synthesis of monolignols

All chemicals and enzymes used in this study, which were of reagent grade and used as-received without further purification prior to use, were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), unless otherwise mentioned.

CA was synthesized from ethyl ferulate [24]. Ethyl ferulate (1.96 g, Tokyo Chemical Industry Co., LTD., Tokyo, Japan) was suspended in 50 mL of distilled toluene with stirring in an ice bath. Diisobutylaluminium hydride (DIBAL, 40 mL) was added dropwise to the suspension. After the reaction, the distilled water was added with vigorous stirring. The resulting mixture was filtrated to obtain the filtrate. To the filtrate, ethyl acetate was added to extract the organic layer. The extraction was repeated 3 times. The extract was evaporated to dryness to give 1.2 g of CA without further purification. The purity of CA in the extract was confirmed by thin-layer chromatography (TLC) and nuclear magnetic resonance (NMR).

SA was synthesized from syringaldehyde and monoethyl malonate [25]. Monoethyl malonate was synthesized according to the method by Xia and Hu [26]. Syringaldehyde (5.0 g) and monoethyl malonate (4.3 g) were dissolved in 33 mL of pyridine and 0.65 mL of piperidine. The mixture was stirred for 1 h at 50°C and heated at 100°C until all syringaldehyde reacted, which was monitored by TLC. The mixture was cooled to 0°C and 33 mL of 12 M hydrochloric acid was added with continuous stirring for 10 min. Then, 100 mL of 0.1 M sodium phosphate buffer (pH 7.0) was added. The resulting mixture was extracted 3 times with ethyl acetate and the extract was evaporated to syrup to give crude ethyl sinapate. The crude ethyl sinapate was further purified through a silica gel column (ethyl acetate/hexane= 1:3) to yield 6.0 g of pure ethyl sinapate. The ethyl sinapate (6.0 g) was dissolved in 25 mL of tetrahydrofuran (THF), and the solution was added dropwise to a suspension of $LiAlH_4$ (1.8 g) in THF (45 mL) containing $AlCl_3$ (1.6 g) at -20°C. The mixture was then heated to 0°C and left to stand with continuous stirring overnight. After completion of the reduction reaction (monitored by TLC), the mixture was added to 200 mL of distilled water and extracted 3 times with ethyl acetate. The ethyl acetate-layer was washed with brine and evaporated to syrup. Crude SA was purified through a silica gel column (methanol/chloroform, 1:9) to give 1.7 g of SA. The purity of SA was confirmed by TLC and NMR.

Extraction of Laccase from crude acetone powder

Laccase from *Rhus vernicifera* was purchased from Sigma-Aldrich (St. Louis, MO, USA). The laccase (5 mg) was suspended in the 1 mL of 0.1 M potassium phosphate buffer (pH 6.5) and homogenized with a homogenizer in a micro tube. After homogenization, the suspension was filtrated with cellulose acetate filter (0.80 μm) to obtain the 1st filtrate (1 mL) and the filtration residue. The filtration residue was washed with 4 mL of 0.1 M potassium phosphate buffer (pH 6.5) and again filtrated to obtain the 2nd filtrate (4 mL). The 1st and the 2nd filtrates were mixed to yield 5 mL of laccase solution.

Enzyme activity test

The enzyme activities of laccase and HRP were determined with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, ε_{420}=3.6×10⁴ $M^{-1}cm^{-1}$) [27], syrinaldazine (ε_{530}=6.5×10⁴ $M^{-1}cm^{-1}$) [28], CA (ε_{264}=1.3×10⁴ $M^{-1}cm^{-1}$) and SA (ε_{272}=8.1×10³ $M^{-1}cm^{-1}$) [18] by monitoring UV/vis spectral changes with a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). One unit was defined as the amount of enzyme that catalyzes a conversion reaction of one micromole of substrate per minute. The enzyme activities were expressed in unit/mg (weight of enzymes).

HRP-catalyzed oxidative conversion of the substrates was determined, using 0.75 μg of HRP. Totally, 2.13 mL of reaction mixture were used. The final concentrations of syringaldazine, ABTS, CA and SA were 0.099, 2.8, 0.071 and 0.071 mM, respectively, with 0.47 μg/L of hydrogen peroxide.

Laccase-catalyzed oxidative conversion of the substrates was determined, using 0.1 mg of laccase. Totally, 3.0 mL of reaction mixture were used. The final concentrations of syringaldazine, ABTS, CA and SA were 0.03, 0.022 0.071 and 0.071 mM, respectively.

Preparation of DHP

CA and SA (100 mg) were separately dissolved in 2 mL of acetone and 48 mL of 0.1 M potassium phosphate buffer (pH 6.5). The solution was added dropwise to 50 mL of a potassium phosphate buffer containing laccase from *R. vernicifera* (2.5×10^{-2} units for CA) for 10 h, using a peristaltic pump (PST-100: Iwaki Co. Ltd., Tokyo Japan) with oxygen bubbling. After the dropwise addition, the mixture was left to stand for 16 h with stirring. The mixture was then centrifuged. The precipitate was washed with ion-exchanged water and lyophilized to yield DHP. The centrifugation supernatant was evaporated to dryness. The resulting solid was extracted with acetone and evaporated to yield the mixture of monolignol and/or DHP oligomers.

In the case of HRP [29], CA and SA (100 mg) were separately dissolved in 2 mL of acetone and 48 mL of 0.1 M sodium phosphate buffer saline (PBS, pH 6.1, containing NaCl 0.8 w/v% and KCl 0.02 w/v%). Hydrogen peroxide (30 wt%, 0.25 mL) was dissolved in another PBS (50 mL). The two buffer solutions containing a monolignol and hydrogen peroxide, respectively, were added dropwise to a PBS (50 mL) containing 5 mg of HRP (3.3×10^3 units for CA) for 10 h. After dropwise addition, the mixture was left to stand for 16 h. The mixture was then centrifuged (3000 *g*). The precipitate was washed with ion-exchanged water and lyophilized to yield DHP. The centrifugation supernatant was evaporated to dryness. The resulting solid was extracted with acetone and evaporated to yield the mixture of monolignol and/or DHP oligomers. The same experiment was separately performed by using a diluted HRP solution (2.5×10^{-2} units for CA).

Gas Chromatographic (GC) analyses of monolignols and DHP oligomers

Part of the mixture of monolignol and/or DHP oligomers from CA and SA was separately dissolved in 1 mL of pyridine with 5 mg of ethyl vanillin as the internal standard. The pyridine solution was subjected to trimethylsilylation with *N,O*-Bis (trimethylsilyl) acetamide. The trimethylsilylated sample was analyzed by GC (GC-4000, GL-Sciences, Tokyo, Japan) with a flame ionization detector (FID). The column InertCap 1701 (30 m × 0.25 mm, 0.25 μm in film thickness) used was. The temperature was initially set at 160°C for 3 min, then raised to 250°C at 9°C/min and maintained for 3 min. The temperatures of the injection port and FID were 260°C and 270°C, respectively. Helium was used as the carrier gas and the total flow was 37.4 mL/min with a split ratio of 20. Makeup gas was composed of hydrogen (35 mL/min), helium (30 mL/min) and air (200 mL/min).

The trimethylsilylated sample was analyzed by GC-MS, using a gas chromatograph (Agilent7890A, Agilent, Santa Clara, CA, USA) with a mass spectrometer (JMS-T100Gv, JEOL, Tokyo Japan). The column used was HP-5 (30m × 0.32 mm, 0.25 mm in film thickness). The temperature was initially set at 100°C for 1.5 min, raised to 300°C at 60°C/min, and then again raised to the final temperature (350°C) at 5°C/min. The temperature of the injection port was 250°C. Helium was used as the carrier gas and the total flow was 1.4 mL/min. Data acquisition was in the range of m/z 20-800.

HSQC-NMR of Acetylated DHP (Ac-DHP)

DHP was dissolved in 1 mL of pyridine and 1 mL of acetic anhydride with stirring, and the mixture was left to stand for 48 h at room temperature. The resulting reaction mixture was poured into toluene and evaporated to dryness. The remaining substances (acetic anhydride, pyridine and acetic acid) other than Ac-DHP were removed under reduced pressure by repeated co-evaporation with toluene until the smell of pyridine was disappeared [30].

The Ac-DHP was dissolved in $CDCl_3$ (D, 99.96%, containing 0.03 v/v% TMS) and analyzed by NMR (Bruker AMX 500)

RESULTS AND DISCUSSION

Enzyme Activity

Enzyme activities of laccase from *R. vernicifera* and HRP were measured spectrophotometrically with syringaldazine, ABTS, CA and SA, to understand the difference in substrate specificity and oxidation ability between the enzymes (Table 1). Laccase had 3.5 times higher activity for syringaldazine than for the ABTS. In the case of HRP, enzyme activity for syringaldazine was only 0.18 times that for ABTS. Laccase and HRP showed different activities for a given substrate. Sterjiades *et al.* [18] reported laccase from sycamore maple showed 3.6 times higher enzyme activity for ABTS than for syringaidazine, which gave a different trend from our result.

Sterjiades *et al.* [18] also reported that laccase from sycamore maple showed 2.8 times higher enzyme activity for SA than for CA, while HRP showed over 10 times higher enzyme activity for CA than for SA. Our results using CA and SA as substrates were essentially consistent with their results, though they were some differences in experimental conditions.

Table 1. Enzyme activities of laccase and HRP against some substrates.

Substrate	Laccase (unit/mg)	HRP (unit/mg)
Syringaldazine	6.0×10^{-3}	0.46×10^{2}
ABTS	1.7×10^{-3}	2.5×10^{2}
CA	2.6×10^{-3}	6.5×10^{2}
SA	1.6×10^{-2}	1.1×10^{2}

Preparation of DHP by Laccase and HRP

Monolignols were oxidized separately by laccase and HRP, and the resulting water-insoluble parts were obtained as DHPs. Yields of DHPs from CA and SA were shown in Table 2. It was reported that laccase and HRP gave only a limited amount of DHPs from SA, while the two enzymes gave a much higher yield of DHPs from CA [7,17,18]. Our results provided a similar result as those in the previous studies [8]. In our experiments, an extraordinarily large difference in enzyme activity was found

Table 2. Yields of DHP from CA and SA by each enzyme.

Enzyme	Enzyme load (units)[1]	Yield of DHP (mg)	
		CA[2]	SA[2]
Laccase	2.5×10^{-2}	38	1.2
HRP	2.5×10^{-2}	0	0
	3.3×10^{3}	86	8.3

[1] Units for coniferyl alcohol (CA) oxidation. ; [2] Substrates of DHP preparation.

between laccase and HRP (Table 1). As anticipated, HRP did not yield DHP at a low charge (0.025 units) condition, under which laccase gave a fair yield of DHP (Table 2). We thus used much higher charge (3.3×10^3 units) of HRP to produce DHP at a favorable yield, in order to further elucidate structural

difference between laccase- and HRP-derived DHPs by 2D-NMR technique.

Analysis of Unreacted Monolignols and DHP Oligomers by GC and GC-MS

The unreacted monolignols and DHP oligomers were analyzed by GC and GC-MS. It was assumed that supernatants of the DHP preparation buffer contained lower molar mass-compounds, compared to the precipitate. We thus monitored if the monolignols were oxidized or remained unreacted in the supernatant to estimate the oxidation ability of the enzymes. Fig 1 (a) and (b) showed the GC spectra of the supernatants, using laccase for CA and SA, respectively. The CA supernatant (Fig 1a) had two main peaks. The peak at the retention time (rt) of 11.0 min was identified as CA. CA remained unreacted in this supernatant. The SA supernatant (Fig 1b) showed a larger number of peaks than the CA supernatant, but did not give a peak of the original SA. These results indicate that SA was oxidized more efficiently than CA by laccase, but no DHP formation from SA was observed, suggesting SA was converted to some unidentified compounds.

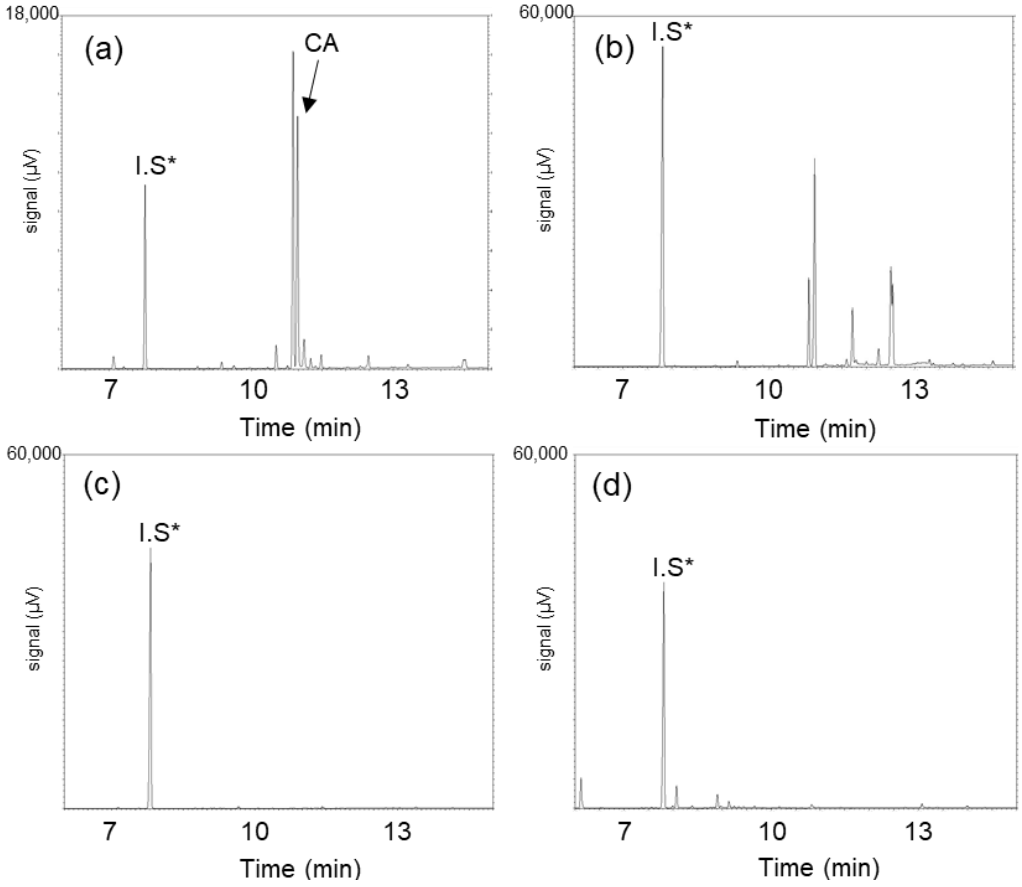

Fig 1. GC chromatograms of supernatant of DHP preparation suspension from CA by laccase (a), from SA by laccase (b), from CA by HRP (c), and from SA by HRP (d). *Ethyl vanillin (retention time: 7.8 min) was used as the internal standard.

In the case of HRP (Fig 1c and 1d), no unreacted monolignols (CA and SA) were observed. These results suggested that both monolignols underwent the HRP-catalyzed initial oxidation, at least completely, under our reaction conditions. The yield of DHP from CA by HRP was 86%, while that from SA was only 8.3%. This result indicated that HRP proceeded polymerization of CA much more effectively than that of SA.

The lower molar mass-compounds in the supernatants from SA were analyzed in more details by GC-MS (Table 3). The molar mass of fully trimethylsilylated SA is calculated to be 354, but in fact such

a compound was not detected by GC-MS. Since the fragment ions and/or molecular ions were detected between 350 to 650 m/z, SA-derived dimeric compounds seemed to be formed. In the case of HRP, only one compound detected was assumed to be a dimer and others were monomeric derivatives. In the case of laccase, three compounds detected were assumed to be dimeric compounds.

In conclusion, both laccase and HRP oxidized monolignols to give DHP oligomers, and also polymerized monolignols to give DHPs under our reaction conditions employed. But the two enzymes seem to have different pathways to convert monolignols to DHP or oligomers.

Table 3. Compounds in DHP preparation suspension from SA detected by GC-MS*.

Number (retention time)	m/z (relative intensity)
Laccase	
1. (4.66)	281 (7), 280 (26), 279 (100)
2. (4.76, 4.79)	443 (5), 442 (19), 441 (42), 440 (100)
3. (4.79)	280 (7), 279 (25), 278 (100)
4. (4.96)	353 (3), 352 (12), 351 (34), 350 (100)
5. (7.96)	624 (2), 623 (10), 622 (42), 621 (100)
6. (9.19)	654 (3), 653 (8), 652 (23), 651 (52), 650 (100)
7. (9.73)	565 (5), 564 (19), 563(44), 562 (100), 500 (2), 499 (6), 498 (14)
HRP	
1. (3.51)	287 (4), 215 (21),214 (8), 213 (8), 201 (4), 200 (18), 199 (100)
2. (3.71)	363 (4), 293 (2), 292 (10), 291 (24), 290 (100), 201 (2)
3. (4.13)	317 (2), 316 (11), 315 (26), 314 (100)
4. (4.38)	329 (2), 328 (10), 327 (27), 326 (100), 312 (2)
5. (4.54, 4.55)	345 (2), 344 (11), 343 (30), 342 (100)
6. (4.55)	371 (2), 370 (11), 369 (30), 368 (100)
7. (4.62)	401 (2), 400 (5), 371 (2), 301 (1), 300 (10), 299 (37), 298 (100), 283 (4)
8. (5.78)	529 (5), 528 (19), 527 (41), 526 (100)

*field ionization method

HSQC NMR of Ac-DHP

As the major interunitary linkages in lignin generally accepted, β-*O*-4, β-β and β-5 bonds are well known [31]. The correlation between proton and carbon at α, β and γ positions of CA-derived DHPs were thus evaluated to confirm the existence of these major linkages [32]. Fig 2 shows the 2D-NMR spectra of Ac-DHPs from CA by laccase and HRP. The Ac-DHP by HRP clearly showed the existence of β-*O*-4, β-5 and β-β bonds. On the other hand, Ac-DHP by laccase also had β-β and β-5 bonds, but the β-*O*-4 bond was missing. These results imply that laccase-catalyzed oxidation gave DHP rich in β-5 and β-β bonds, while HRP-catalyzed oxidation produced DHP rich in β-*O*-4 bond. Our results look consistent with those by Okusa *et al*., who reported laccase from *R. vernicifera* produced dimeric compounds from CA, which was rich in pinoresinol and phenylcoumaran, but not in β-*O*-4 bond [22].

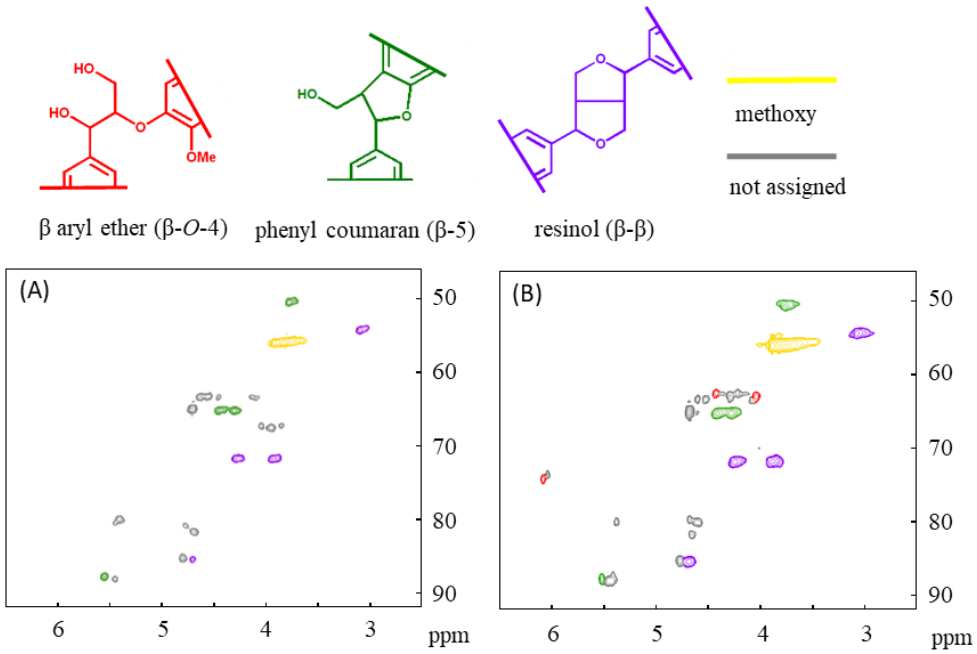

Fig 2. Aliphatic region of HSQC spectra. Ac-DHP from CA by laccase (A) and from CA by HRP (B).

CONCLUSIONS

1. SA showed much less reactivity than CA in the formation of DHP when oxidized and/or polymerized *via* laccase from *Rhus vernicifera*, as well as *via* HRP.
2. Laccase from *R. vernicifera* gave DHP and/or its oligomers rich in β-β and β-5 linkages, but not in β-*O*-4 bond, while HRP gave DHP containing these three linkages, when these enzymes were used for DHP production.

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