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Takayuki Kobayashi · Hiroyasu Taguchi
Mikiji Shigematsu · Mitsuhiko Tanahashi

Substituent effects of 3,5-disubstituted *p*-coumaryl alcohols on their oxidation using horseradish peroxidase–H_2O_2 as the oxidant

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Abstract The consumption rates of three monolignols (*p*-coumaryl, coniferyl, and sinapyl alcohols) and eight analogues using horseradish peroxidase (HRP)–H_2O_2 as an oxidant were measured and compared with the anodic peak potentials thereof measured with cyclic voltammetry. 3-Monosubstituted *p*-coumaryl alcohols, i.e., 3-methoxy-, 3-ethoxy-, 3-*n*-propoxy-, and 3-*n*-butoxy-*p*-coumaryl alcohols, had faster reaction rates than *p*-coumaryl alcohol. This is most probably due to the electron-donating effect of alkoxyl groups. However, the reaction rates gradually decreased with an increase in the molecular weight of the alkoxyl groups. Furthermore, *t*-butoxyl group, which is a very bulky substituent, caused an extreme reduction in the reaction rate, even though its electron-donating effect was almost the same as that of other alkoxyl groups. The reaction rates of 3,5-disubstituted *p*-coumaryl alcohols, especially 3,5-dimethyl-*p*-coumaryl alcohol, were very low compared with 3-monosubstituted *p*-coumaryl alcohols. These results suggest that there are three main factors of hindrance during the approach of monolignols to the active site of HRP. First, from the results of 3-monoalkoxy-*p*-coumaryl alcohols, it was suggested that the volume of substituents could decrease their oxidation rates. Second, from the results of 3,5-disubstituted *p*-coumaryl alcohols, it was suggested that local steric hindrance by the amino residues quite near the heme decreased the oxidation rates. Third, from the results of the substrates with hydrophobic substituents at their 3,5-positions, we suggested that hydrophilicity near heme would decrease their oxidation rates.

Key words Horseradish peroxidase · Lignin · *p*-Coumaryl alcohol derivative · Anodic peak potential

T. Kobayashi (✉) · H. Taguchi · M. Shigematsu · M. Tanahashi
Science of Biological Resources, United Graduate School of Agricultural Science, Gifu University, Gifu 501-1193, Japan
Tel. +81-58-293-2922; Fax +81-58-293-2922
e-mail: dxv3688@cc.gifu-u.ac.jp

Introduction

Lignins are located in the primary walls, secondary walls, and middle lamella of plants higher than ferns. They give plants mechanical support and defend against fungi and pathogens. Studies on lignification of cell walls have been performed, yet many points still remain unclear. For instance, as far as we know, the synthesis of dehydrogenation polymer (DHP) rich in sinapyl alcohol has not yet been accomplished efficiently in water milieu, although there is a report on the synthesis of DHP, which has β-*O*-4 bonds that are common in native lignins, in dioxane using iron (III) chloride as an oxidant.[1] Furthermore, Aoyama et al.[2] and Sasaki et al.[3] reported that high molecular weight lignin rich in sinapyl alcohol was synthesized by a poplar cell wall peroxidase that is specific for sinapyl alcohol, but its chemical structure was not clear. Essentially, we do not have a unified idea about the mechanism of lignification of cell walls. This, however, seems to be unavoidable, because there are many factors to be considered in order to elucidate the mechanism of lignification, such as solvent effects,[1] pH effect,[4] the kind of enzymes associated with lignification,[5,6] interactions among monolignols,[7] and rates of supply of substances.[8] We are also of the view that there is a need to accumulate basic knowledge about the oxidation rate of monolignols with enzymes and/or association constants between monolignols and horseradish peroxidase (HRP) as clues for the elucidation of lignification in cell walls. Carrying out detailed investigation would be useful not only for elucidation of the influence of interaction among monolignols on lignification but also for elucidation of the reaction of HRP with dimers, trimers, and polymers. In this study, we attempted to obtain knowledge on the mechanism of the oxidation of monolignols by HRP-H_2O_2.

Peroxidases have been considered as a lignification-catalyzing enzyme in cell walls, and above all HRP-H_2O_2 has been used as an oxidant for the synthesis of DHP. A well-known scheme for oxidation of substrate compound AH with HRP-H_2O_2 is shown as follows:[9–11]

$$\left[\mathrm{Fe(III)(Porp)}\right] + \mathrm{H_2O_2} \rightarrow \left[\mathrm{Fe(IV) = O(Porp)^{+}}\right] + \mathrm{H_2O} \quad (1)$$

$$\left[\mathrm{Fe(IV) = O(Porp)^{+}}\right] + \mathrm{AH} \rightarrow \mathrm{H^{+}\left[Fe(IV) = O(Porp)\right]} + \mathrm{A\cdot} \quad (2)$$

$$\mathrm{H^{+}\left[Fe(IV) = O(Porp)\right]} + \mathrm{AH} \rightarrow \left[\mathrm{Fe(III)(Porp)}\right] + \mathrm{A\cdot} + \mathrm{H_2O} \quad (3)$$

where [Fe(III)(Porp)], [Fe(IV) = O(Porp)$^{+}$], and H^{+}[Fe(IV) = O(Porp)] show native, compound I, and compound II of HRP, respectively, and porp represents a porphrin ring. Regarding HRP compound I, Dominique and Dunford[12] reported that its oxidation rate for meta or para substituted phenol depends on the magnitude of the electronic effects of substituents on the benzene ring. For compound II, it was reported that the reaction rate was slower by a factor of ten compared with that of compound I, and that an electron was fed to the ferryl group, while an electron was fed to the electron-deficient porphyrin π-cation radical of compound I.[13] There are many reports on the oxidation of meta or para substituted phenols by HRP-H_2O_2, but few on ortho substituted phenols. In this study, we reacted three monolignols and eight kinds of 3-monosubstituted and/or 3,5-disubstituted *p*-coumaryl alcohol with HRP–H_2O_2. Furthermore, we determined the order of oxidizability of these compounds with cyclic voltammetry, which we previously determined by computational calculation by MOPAC2000.[14] By comparing these results, we examined the oxidation of monolignols with HRP-H_2O_2 in terms of steric and electronic effects of substituents.

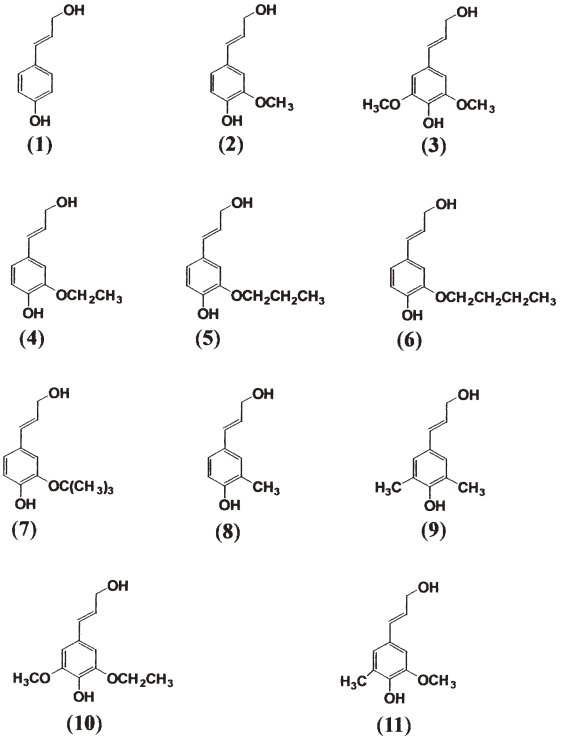

Fig. 1. The structures of monolignols and *p*-coumaryl alcohol derivatives used in this study. (**1**) *p*-Coumaryl alcohol, (**2**) Coniferyl alcohol, (**3**) Sinapyl alcohol, (**4**) 3-Ethoxy-*p*-coumaryl alcohol, (**5**) 3-*n*-Propoxy-*p*-coumaryl alcohol, (**6**) 3-*n*-Butoxy-*p*-coumaryl alcohol, (**7**) 3-*t*-Butoxy-*p*-coumaryl alcohol, (**8**) 3-Methyl-*p*-coumaryl alcohol, (**9**) 3,5-Dimethyl-*p*-coumaryl alcohol, (**10**) 3-Ethoxy-5-methoxy-*p*-coumaryl alcohol, (**11**) 3-Methoxy-5-methyl-*p*-coumaryl alcohol

Materials and methods

Materials

The chemical structures of the compounds used in this study are shown in Fig. 1. Monolignols **1**–**3** were synthesized by the method of Freudenberg and Hübner.[15] The other alcohols **4**–**11** were synthesized from the corresponding *p*-hydroxybenzaldehyde derivatives by the same method used for monolignols.

3-Ethoxy-, 3-*n*-propoxy-, and 3-*n*-butoxy-*p*-hydroxybenzaldehydes were synthesized from *o*-ethoxyphenol, *o*-*n*-propoxyphenol, and *o*-*n*-butoxyphenol, respectively: 3 g of each phenol was dissolved into water (100 ml) containing fourfold molar excess of sodium hydroxide for each phenol, and chloroform was added into the solution slowly at ca. 80°C. After checking the completion of the reaction, the solution was neutralized with 6 mol dm^{-3} aqueous HCl, and each benzaldehyde was separated into ethyl acetate. After the solvent was evaporated to dryness, the sample was subjected to column chromatography on silica gel. *o*-Ethoxyphenol, *o*-*n*-propoxyphenol, and *o*-*n*-butoxyphenol were synthesized by alkoxylation of catechol using ethyl iodide, *n*-propyl bromide, and *n*-butyl bromide, respectively, with potassium carbonate in acetone at room temperature. After neutralization of the solution, ethyl acetate was added to the solution, and ethyl acetate layer was separated. After the ethyl acetate solvent was evaporated to dryness, each sample was purified by column chromatography on silica gel.

3-Methyl-, 3,5-dimethyl-, and 3-*t*-butoxy-*p*-hydroxybenzaldehydes were synthesized from *o*-cresol, 2,6-dimethylphenol, and *o*-*t*-butoxyphenol, respectively, by the same method used for 3-ethoxy-*p*-hydroxybenzaldehyde. *o*-*t*-Butoxyphenol was synthesized from catechol with isobutene. Catechol was dissolved into chloroform and three drops of concentrated H_2SO_4 were added into the solution, after which isobutene was bubbled into the solution. After confirming the end of the reaction by thin layer chromatography, the solution was concentrated using a rotary evaporator and the obtained *o*-*t*-butoxyphenol was purified by column chromatography on silica gel.

3-Ethoxy-5-methoxy-*p*-hydroxybenzaldehyde and 3-methoxy-5-methyl-*p*-hydroxybenzaldehyde were synthesized from 3-ethoxy-*p*-hydroxybenzaldehyde and 3-methyl-*p*-hydroxybenzaldehyde, respectively, via 3-ethoxy-5-iodo-*p*-hydroxybenzaldehyde and 3-iodo-5-methyl-*p*-hydroxybenzaldehyde[16] according to the method of Pepper and MacDonald.[17] Synthesized monolignol ana-

logues were identified using ^{1}H nuclear magnetic resonance (NMR) spectroscopy (Varian INOVA 400 or 500) with tetramethylsilane (TMS) as internal standard. The assignment of each compound is as follows: 3-ethoxy-*p*-coumaryl alcohol (**4**): ^{1}H-NMR: δ 1.46 (t, 3H, J = 7.0, -OCH$_2$<u>CH$_3$</u>), 4.13 (q, 2H, J = 7.0, -O<u>CH$_2$</u>CH$_3$), 4.30 (d, 2H, J = 5.9, Cγ H), 5.70 (s, 1H, phenolic OH), 6.23 (dt, 2H, J = 5.9, J = 15.7, CβH), 6.52 (d, 2H, J = 16.1, CαH), 6.85–6.91 (3H, aromatic H). 3-*n*-Propoxy-*p*-coumaryl alcohol (**5**): ^{1}H-NMR: δ 1.06 (t, 3H, J = 7.3, -OCH$_2$CH$_2$<u>CH$_3$</u>), 1.91 (sextet, 2H, J = 7.3, -OCH$_2$<u>CH$_2$</u>CH$_3$), 4.02 (t, 2H, J = 6.6, -O<u>CH$_2$</u>CH$_2$CH$_3$), 4.29 (d, 2H, J = 5.9, Cγ H), 5.67 (s, 1H, phenolic H), 6.21 (dt, 2H, J = 5.9, J = 15.7, Cβ H), 6.53 (d, 2H, J = 15.7, CαH), 6.87–6.91 (3H, aromatic H). 3-*n*-Butoxy-*p*-coumaryl alcohol (**6**): ^{1}H-NMR: δ 1.00 (t, 3H, J = 5.0, -OCH$_2$CH$_2$CH$_2$<u>CH$_3$</u>), 1.51 (sextet, 2H, J = 15, -OCH$_2$CH$_2$<u>CH$_2$</u>CH$_3$), 1.82 (quintet, 2H, J = 6, -OCH$_2$<u>CH$_2$</u> CH$_2$CH$_3$), 4.06 (t, 2H, J = 6.6, -O<u>CH$_2$</u>CH$_2$CH$_3$), 4.29 (d, 2H, J = 5.9, CγH), 5.66 (s, 1H, phenolic OH), 6.21 (dt, 2H, J = 5.9, J = 15.7, CβH), 6.53 (d, 2H, J = 15.7, CαH), 6.87–6.91 (3H, aromatic H). 3-*t*-Butoxy-*p*-coumaryl alcohol (**7**): ^{1}H-NMR: δ 1.43 [s, 9H, -OC(CH$_3$)$_3$], 4.28 (d, 2H, J = 5.9, CγH), 5.77 (s, 1H, phenolic OH), 6.19 (dt, 2H, J = 5.9, J = 15.7, CβH), 6.50 (d, 2H, J = 15.7, CαH), 6.88–7.08 (3H, aromatic H). 3-Methyl-*p*-coumaryl alcohol (**8**): ^{1}H-NMR: δ 2.25 (s, 3H, -CH$_3$), 4.29 (d, 2H, J = 5.9, CγH), 4.94 (s, 1H, phenolic OH), 6.22 (dt, 2H, J = 6.0, J = 15.7, CβH), 6.52 (d, 2H, J = 16.1, CαH), 6.73 [d, 1H, J = 8.1, aromatic (C5) H], 7.18 [dd, 1H, J = 2.2, J = 8.1, aromatic (C6) H], 7.17 [s, 1H, aromatic (C2) H]. 3,5-Dimethyl-*p*-coumaryl alcohol (**9**): ^{1}H-NMR: δ 2.24 (s, 6H, -CH$_3$), 4.28 (d, 2H, J = 5.9, CγH), 4.69 (s, 1H, phenolic OH), 6.22 (dt, 2H, J = 6.0, J = 16.1, CβH), 6.49 (d, 2H, J = 15.7, CαH), 7.03 (2H, aromatic H). 3-Ethoxy-5-methoxy-*p*-coumaryl alcohol (**10**) (acetate): ^{1}H-NMR: δ 1.37 (t, 3H, J = 7.0, -OCH$_2$<u>CH$_3$</u>), 2.10 (s, 3H, aliphatic acetate), 2.32 (s, 3H, aromatic acetate), 3.82 (s, 3H, -OCH$_3$), 4.05 (q, 2H, J = 7.0, -OCH$_2$<u>CH$_3$</u>), 4.71 (d, 2H, J = 6.6, CγH), 6.22 (dt, 2H, J = 6.4, J = 15.7, CβH), 6.57 (d, 2H, J = 15.7, CαH), 6.62 (2H, aromatic H). 3-Methoxy-5-methyl-*p*-coumaryl alcohol (**11**) (acetate): ^{1}H-NMR: δ 2.10 (s, 3H, aliphatic acetate), 2.16 (s, 3H, -CH$_3$), 2.33 (s, 3H, aromatic acetate), 3.82 (s, 3H, -OCH$_3$), 4.71 (dd, 2H, J = 6.4, J = 1.1, CγH), 6.22 (dt, 2H, J = 6.4, J = 15.8, CβH), 6.58 (d, 2H, J = 15.8, Cα-H), 6.82 (dd, H, J = 1.8, aromatic H), 6.84 (dd, H, J = 1.8, aromatic H).

Measurement of anodic peak potentials of compounds by cyclic voltammetry

Approximately 0.1 mmol of each compound was dissolved in 0.4 mol dm^{-3} anhydrous tetraethylammonium tetrafluoroborate acetonitrile solution. For measurement of anodic peak potential (E_{pa}) of each compound in the phenolate state, 0.1 mmol of tetramethylammonium hydroxide pentahydrate was added. A platinum electrode with a 0.3 mm diameter, which was polished before each voltammogram with abrasive film (grain size 3 μm), a platinum wire electrode, and a saturated KCl Ag/AgCl electrode were used as the working, counter, and reference electrodes, respectively. The cyclic voltammogram was measured using a HSV-100 (Hokutodenko, JAPAN) at 25°C. In all measurements, the sweep rate was 100 mV s^{-1}. Before and after the measurements, the reference electrode was calibrated by the standard potential of the ferrocene/ferrocenium couple.[18] The conversion formula used in this study was as follows:

$$E_{pa}\ \text{vs NHE} = E_{pa}\ \text{vs Ag/AgCl} - E_{1/2}\left(\text{Fc/Fc}^+\right)\ \text{vs Ag/AgCl} + E^0\left(\text{F}_c/\text{Fc}^+\right)\ \text{vs NHE} \quad (4)$$

where E_{pa} is the anodic peak potential of a compound, $E_{1/2}$(Fc/Fc$^+$) is the half-wave potential of ferrocene/ferrocenium, and NHE signifies the normal hydrogen electrode. $E_{1/2}$(Fc/Fc$^+$) vs Ag/AgCl was measured to be 0.456 ± 0.003 V in our study, and E^0 (Fc/Fc$^+$) vs NHE was 0.400 V.

Oxidation of monolignols and their analogues

Approximately 100 mmol of each monomer was dissolved in 20 ml of potassium phosphate buffer (0.1 mol dm^{-3}, pH 7.4). HRP (type VII) obtained from Sigma [St. Louis, MO (3.3 ± 0.2) × 10^{-6} kat (mean ± standard deviation)] was added, and then the reaction was started by adding 50 μl of 3% H_2O_2 solution at 25°C. At definite time intervals (0, 1, 2, 5, 10, 15 min), 2 ml of solution was sampled into vials containing ascorbic acid, which was used to stop the reaction. Then, 2 ml of ethyl acetate containing vanillin acetate, acting as an internal standard, was added to the vials. Following shaking, 1 ml of solution was sampled from the ethyl acetate layer into a pear-shaped flask. After evaporating the ethyl acetate, acetylation was carried out by the addition of pyridine and acetic anhydride. Samples were subjected to gas chromatography (GC) analysis to measure the concentration of compounds. GC analyses of compounds were performed on a Hitachi 263-30 using a capillary column, DB-5 (film thickness 1 μm; column dimensions 30 m × 0.53 mm), with N_2 as carrier gas. Column temperature was programmed from 80° to 260°C at an increasing rate of 15°C min^{-1}.

3-Methyl- and 3,5-dimethyl-*p*-coumaryl alcohols showed low solubility in water; thus, the reaction rates of these compounds could not be obtained by GC. Therefore, reaction rates of these compounds were measured with ultraviolet (UV) spectroscopy (Jasco V-550 UV/Vis) at 25°C and the relative values based on *p*-coumaryl alcohol (**1**) are used in the discussion. Three milliliters of 0.04–0.05 mmol dm^{-3} aqueous solution of the compound was added to a quartz cell and HRP [(3.3 ± 0.2) × 10^{-6} kat (mean ± standard deviation)] was added. To start the reaction, 25 μl of 3% H_2O_2 solution was added. The decrease in substrate was calculated from the decrease of absorbance at 260 nm.

Measurement of the proportion of dilignols produced by enzymatic dehydrogenation polymerization

Ten milligrams of each *p*-coumaryl alcohol derivative was dissolved into 0.1 M potassium phosphate buffer (pH 7.4) and 50 μl of 7.2 μM HRP solution was added. The concentration of HRP was determined by using $\varepsilon_{403} = 1.02 \times 10^5 M^{-1} cm^{-1}$. About a half molar equivalent of H_2O_2 with respect to each derivative was added to the buffer solution and the resulting solution was stirred for 12 h. The solution was extracted with ethyl acetate (3×100 ml) and the ethyl acetate layer was dried with sodium sulfate. Before the extraction, a known volume of vanilline acetate was added into the solution as an internal standard for quantification. After evaporation of ethyl acetate under reduced pressure, samples were analyzed by ^{1}H-NMR in acetone-d_6 with TMS as the internal standard. The yields of β-5, β-*O*-4, and resinol dimers were determined by the peak integral of the α-methene proton of each dilignol based on that of the aldehyde proton of vanilline acetate.

Results and discussion

E_{pa} values of monolignols and analogues in phenol and phenolate states

To determine the oxidizability of each compound, we used cyclic voltammetry to measure the redox potential. As typical examples, cyclic voltammograms of coniferyl alcohol (**2**) in phenol and phenolate states are shown in Fig. 2. Both voltammograms showed that the oxidation is irreversible, because an anodic peak was observed but there was no appearance of a cathodic peak. From these results, it could be inferred that the coniferyl alcohol radicals formed by oxidation near the working electrode were coupled into dimers so fast with respect to sweep rate that the cathodic peak could not be observed. Hapiot and Pinson[19] measured one-electron redox potentials for the oxidation of coniferyl alcohol (**2**) and analogues with an ultramicroelectrode (10 μm diameter) at a fast scan rate of up to 20000 V s^{-1}. Because the scan rate of the voltammeter used in the present study was limited to 100 mV s^{-1}, irreversible voltammograms were observed. Therefore, oxidizabilities of compounds were estimated by E_{pa} values rather than half-wave potentials. The measured E_{pa} of each compound is arranged in Table 1.

First, our discussion focuses on the differences between the phenolate and phenol forms. Each E_{pa} in the phenolate form shifted by about -1 V, compared with that in the phenol form. These results suggest that HRP preferably oxidizes compounds in their phenolate form. Furthermore, the distribution of the E_{pa} of phenolates was wider than that of phenols. As an example, the difference in E_{pa} (ΔE_{pa}) between sinapyl (**3**) and coniferyl (**2**) alcohols in their phenol and phenolate forms were -27 mV and -242 mV, respectively. The same phenomenon was observed in our pervious study of the HOMO levels of monolignols calculated with MOPAC2000.[14]

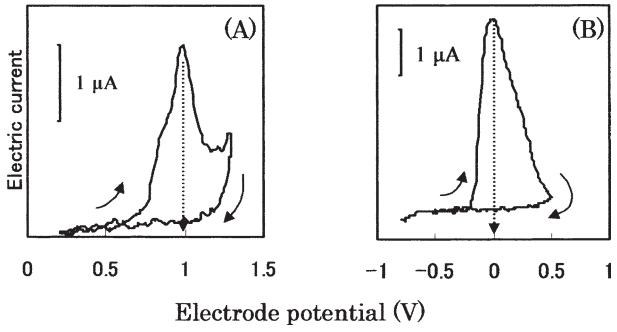

Fig. 2. Cyclic voltammograms of coniferyl alcohol in the phenol form (**A**) and phenolate form (**B**). **A** Solvent: 0.4 mol dm^{-3} NEt_4BF_4 in acetonitrile; concentration of coniferyl alcohol: 0.8 mol dm^{-3}; scan rate 100 mV s^{-1}. **B** Solvent: 0.4 mol dm^{-3} NEt_4BF_4 in acetonitrile with $N(CH_3)_4OH \cdot 5H_2O$; concentration of coniferyl alcohol; 0.03 mol dm^{-3}; scan rate, 100 mV s^{-1}. *Solid arrows* show sweep direction. *Dotted arrows* indicate anodic peak potentials

Table 1. Anodic peak potentials of monolignols and analogues in the phenol or phenolate state

Monolignols and analogues	Group	Anodic peak potential[a] (mV vs NHE)	
		Phenol	Phenolate
p-Coumaryl alcohol (**1**)		1195 ± 10 (6)	180 ± 15 (10)
Coniferyl alcohol (**2**)	A	1007 ± 2 (6)	−15 ± 4 (5)
Sinapyl alcohol (**3**)	C	980 ± 4 (6)	−257 ± 5 (6)
3-Ethoxy-*p*-coumaryl alcohol (**4**)	A	1056 ± 15 (4)[b]	−60 ± 2 (4)[b]
3-*n*-Propoxy-*p*-coumaryl alcohol (**5**)	A	1069 ± 6 (4)[b]	−60 ± 2 (4)[b]
3-*n*-Butoxy-*p*-coumaryl alcohol (**6**)	A	1018 ± 5 (5)	−12 ± 6 (7)
3-*t*-Butoxy-*p*-coumaryl alcohol (**7**)	A	1087 ± 6 (6)	−52 ± 4 (3)
3-Methyl-*p*-coumaryl alcohol (**8**)	B	1176 ± 16 (6)	72 ± 16 (11)
3,5-Dimethyl-*p*-coumaryl alcohol (**9**)	B C	1026 ± 4 (5)	−116 ± 11 (4)
3-Ethoxy-5-methoxy-*p*-coumaryl alcohol (**10**)	C	1001 ± 7 (4)	−247 ± 5 (4)
3-Methoxy-5-methyl-*p*-coumaryl alcohol (**11**)	C	992 ± 2 (6)	−247 ± 4 (4)

Grouping: A, 3-mono alkoxyl-*p*-coumaryl alcohols; B, substrates having only methyl groups; C, 3,5-disubstituted-*p*-coumaryl alcohols
NHE, natural hydrogen electrode
[a] Results given as mean ± standard error (number of measurements)
[b] Measured at 20°C

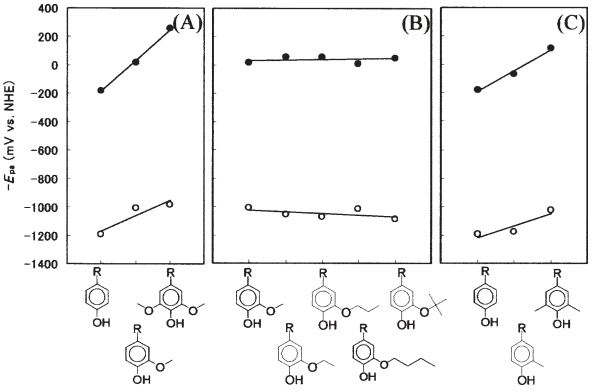

Fig. 3. Effect of substituents on the anodic peak potentials (E_{pa}) of monolignols (**A**), mono alkoxyl series (group A) (**B**), methyl series (group B) (**C**). *Lines* are to guide reader's eye. High $-E_{pa}$ value means high oxidizability. *Filled circles*, phenolate form; *open circles*, phenol form; R-, -CH=CHCH$_2$OH

The apparent redox potential (E^0_{app}) and dissociation constants for oxidant (K_O) or reductant (K_R) of each compound are related by the equation,

$$E^0_{app} = E^0 + \frac{RT}{nF}\ln\frac{\frac{a_L}{K_R} + 1}{\frac{a_L}{K_o} + 1} \quad (5)$$

where E^0 is the standard redox potential, n is the number of electrons, and a_L is the activity of the ligand, which is a proton in this study. If a_L is sufficiently larger than K_O and K_R, the equation is reduced to

$$E^0_{app} = E^0 + \frac{RT}{nF}\ln\frac{K_O}{K_R} \quad (6)$$

In this case, E^0_{app} shows the standard redox potential of the pair of complexes. From Eq. 6 it is clear that the difference between E^0 and E^0_{app} is decided by the dissociation constant ratio between the proton–oxidant complex and the proton–reductant complex. Therefore, the small distribution width of E_{pa} values in the phenol form may be attributed to the differences in the K_O/K_R of each compound. Furthermore, from Fig. 3 the deviations of E_{pa} values for the phenol form from a straight line were larger than those for the phenolate form.

Next, our discussion focuses on E_{pa} values of compounds in the phenolate form. From Table 1, it was observed that E_{pa} values decreased with methoxyl or methyl substitution at the 3- or 3,5-positions of *p*-coumaryl alcohol. The electron-donating effect of the methoxyl and methyl groups can explain these results. Comparing 3-monoalkoxyl- and 3-methyl-*p*-coumaryl alcohols (group A and **8**) with *p*-coumaryl alcohol (**1**), the ΔE_{pa} values were 195–249 mV and 108 mV, respectively. This indicates that the methoxyl group has a greater electron-donating effect than the methyl group. In the group A, the E_{pa} values showed almost the same values in spite of their different chain lengths (Fig. 3B). Regarding the 3,5-disubstituted compounds, the effect of substituents on E_{pa} values increased with the number of substituents. As an example of the case of methoxyl groups, ΔE_{pa} for *p*-coumaryl alcohol (**1**) to coniferyl alcohol (**2**) was 195 mV and ΔE_{pa} for coniferyl alcohol (**2**) to sinapyl alcohol (**3**) was 242 mV (Fig. 3A). For the methyl groups, ΔE_{pa} for *p*-coumaryl alcohol (**1**) to 3-methyl-*p*-coumaryl alcohol (**8**) was 108 mV, and ΔE_{pa} for 3-methyl-*p*-coumaryl alcohol (**8**) to 3,5-dimethyl-*p*-coumaryl alcohol (**9**) was 188 mV (Fig. 3C). Lind et al.[20] reported that there were good correlations between one-electron reduction potentials of 4-substituted phenoxyl radicals in water and Hammett σ values. From that report, methoxyl and methyl groups could be understood to be electron-donating groups at the 4-position of phenols. In our study, it was shown that the methoxyl and methyl groups showed electron-donating effects at the 3- and/or 5-position of *p*-coumaryl alcohol derivatives.

Ratios of dimers yielded by the reaction of monolignols and analogues with HRP–H_2O_2

We investigated the molar ratios of dilignols produced via enzymatic dehydrogenative polymerization of *p*-coumaryl alcohol derivatives by using ^{1}H-NMR and the results are tabulated in Table 2. Compared with β-*O*-4 dimer, the proportions of resinols obtained were higher in the reaction of 3,5-disubstituted derivatives. The proportion of β-*O*-4 dimer in the reaction of 3,5-dimethyl-*p*-coumaryl alcohol (**9**) was the highest among the 3,5-disubstituted-*p*-coumaryl alcohols (group C). Furthermore, the proportion of β-*O*-4 dimer in the reaction of 3-methyl-*p*-coumaryl alcohol (**8**) was higher than those for substrates in group A. These results were considered to be due to the smaller $-$M effect of the methyl group compared with those of alkoxyl groups, as stated in our previous report[1] in which the influence of the steric factor on the proportion of dilignols was also not seen.

Regarding group A, the proportion of β-5 dimer was the highest among the three dimers and the proportions of corresponding dimers produced were very similar. The fact that each monomer produced a corresponding resinol structure showed that each monomer was consumed via one-electron oxidation. High yields for all substrates also indicated that hydrogen peroxide was consumed for producing dimers and not for other reactions. Thus it was confirmed that the oxidation of monomers with HRP–H_2O_2 could be accurately measured at this point in the experiments to measure the reactivity of each monomer.

Reactivity of monolignols and analogues with HRP–H_2O_2

Figure 4 shows the concentration changes of coniferyl alcohol (**2**) during oxidation with HRP–H_2O_2 as an oxidant. From this figure, the decreasing rate of coniferyl alcohol was almost constant and the same in all three experiments up to 300 s. Thus, the reaction rate was considered to be maximal in this concentration range. After 300 s the rate became slower and deviated from the initial rate line. This was possibly due to a lack of H_2O_2, which was the same

Table 2. Yields of dilignols produced by enzymatic dehydrogenative polymerization of each *p*-coumaryl alcohol derivative in 0.1 M potassium phosphate buffer (pH 7.4)

Monolignols and analogues	H_2O_2 : alcohol ratio (mol/mol)	Yields of three dilignols in mol%[a]			
		Resinols	β-*O*-4 Dimers	β-5 Dimers	Total
p-Coumaryl alcohol (**1**)	0.5	30 (36)	21 (25)	32 (39)	82
Coniferyl alcohol (**2**)	0.5	24 (25)	26 (27)	48 (49)	98
Sinapyl alcohol (**3**)	0.5	99 (99)	1 (1)	–	100
3-Ethoxy-*p*-coumaryl alcohol (**4**)	0.45	20 (23)	18 (22)	46 (55)	84
3-*n*-Propoxy-*p*-coumaryl alcohol (**5**)	0.45	15 (24)	12 (19)	36 (57)	64
3-*n*-Butoxy-*p*-coumaryl alcohol (**6**)	0.45	19 (31)	10 (16)	34 (54)	62
3-*t*-Butoxy-*p*-coumaryl alcohol (**7**)	0.45	15 (25)	16 (27)	28 (48)	59
3-Methyl-*p*-coumaryl alcohol (**8**)	0.45	26 (35)	27 (36)	22 (29)	75
3,5-Dimethyl-*p*-coumaryl alcohol (**9**)	0.4	49 (74)	17 (26)	–	66
3-Methoxy-5-methyl-*p*-coumaryl alcohol (**11**)	0.5	84 (82)	18 (18)	–	102

The figures in parentheses are mole percentages among the three dimeric products
[a] These values were calculated by taking dimeric products as two phenols; yields of dimeric products = (dimeric products × 2)/alcohols oxidized as a starting material

Table 3. Rates of consumption of monolignols and analogues measured by gaschromatography (GC) or ultraviolet spectroscopy (UV)

Monolignols and analogues	Group	Rate (μmol s^{-1}) measured by GC[a]	Relative rate measured by UV
p-Coumaryl alcohol (**1**)		38 ± 6 (11)	1
Coniferyl alcohol (**2**)	A	160 ± 10 (8)	6.1
Sinapyl alcohol (**3**)	C	14 ± 0.4 (4)	0.04
3-Ethoxy-*p*-coumaryl alcohol (**4**)	A	120 ± 8 (4)	–
3-*n*-Propoxy-*p*-coumaryl alcohol (**5**)	A	92 ± 4[b]	–
3-*n*-Butoxy-*p*-coumaryl alcohol (**6**)	A	52 ± 6[c]	–
3-*t*-Butoxy-*p*-coumaryl alcohol (**7**)	A	1.1 ± 0.5[c]	–
3-Methyl-*p*-coumaryl alcohol (**8**)	B	–	0.44
3,5-Dimethyl-*p*-coumaryl alcohol (**9**)	B C	–	Negligible
3-Ethoxy-5-methoxy-*p*-coumaryl alcohol (**10**)	C	20 ± 10	–
3-Methoxy-5-methyl-*p*-coumaryl alcohol (**11**)	C	115 ± 7	–

[a] Mean ± standard error (number of measurements)
[b] Initial concentration: 0.5 mM
[c] Initial concentration: 0.25 mmol dm^{-3}

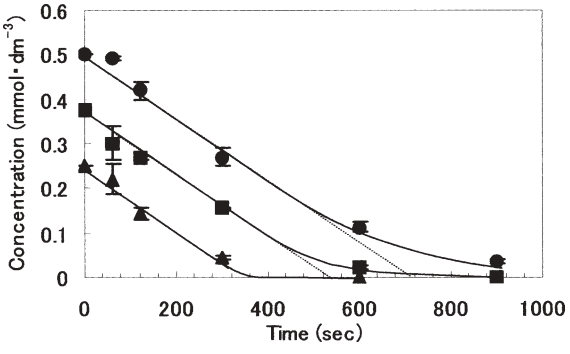

Fig. 4. Reaction curves for oxidation of coniferyl alcohol at different initial concentrations using HRP–H_2O_2 as the oxidant. *Circles*, initial concentration 0.5 mmol dm^{-3}, n = 2; *squares*, initial concentration 0.375 mmol dm^{-3}, n = 4; *triangles*, initial concentration 0.25 mmol dm^{-3}, n = 3. *Error bars* show standard error

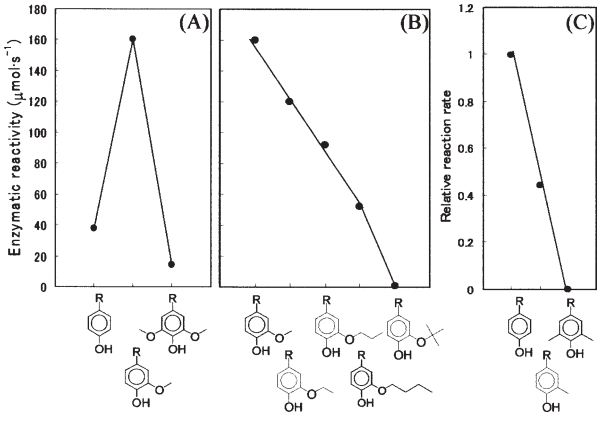

Fig. 5. Effect of substituents on the enzymatic reaction rate for **A** monolignols, **B** alkoxyl series (group A), and **C** methyl series (group B). *Lines* are to guide reader's eye. R-, -CH=CHCH$_2$OH

in the three experiments (0.25 mmol). Because coniferyl alcohol had the fastest reaction rate among all compounds used in this study, we inferred that the rates of other substrates obtained from the initial slope were also the maximal rates.

The E_{pa} of sinapyl alcohol (**3**) was the lowest among the three monolignols, as determined by cyclic voltammetry in acetonitrile. This indicates that sinapyl alcohol was the most easily oxidized substrate of the three monolignols in acetonitrile. The relation between the reaction rates of coniferyl alcohol (**2**) and *p*-coumaryl alcohol (**1**) with HRP qualitatively agreed with that of the E_{pa} values. However, as reported and shown in Table 3 and Fig. 5A, the oxidation rate

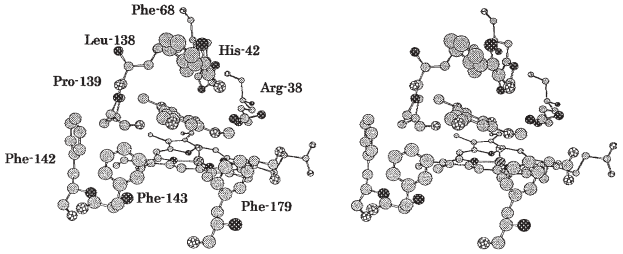

Fig. 6. Stereoview of the active site of horseradish peroxidase quoted from Protein Data Bank (ID: 7atj) with sinapyl alcohol (*center*). The distance between the oxygen of the phenolic hydroxide of sinapyl alcohol and the nitrogen of Arg-38 nearest to sinapyl alcohol or Fe are 2.9 or 5.6 Å, respectively. The distance between methoxyl oxygen of sinapyl alcohol and the nitrogen of Arg-38 is 3.2 Å

of sinapyl alcohol (**3**) was the smallest among the three monolignols. Dominique and Dunford[12] reported on the reactivity of meta and para substituted phenols with HRP compound I and showed the relationship between reactivity and Hammett σ values. Furthermore, Dunford and Adeniran[13] provided a report on HRP compound II dealing with syringic acid and *p*-hydroxybenzoic acid. In their report it was mentioned that syringic acid was much more affected by steric factors than *p*-hydroxybenzoic acid, although details and grounds for this conclusion were not described. The results in this experiment on the reaction rates and the E_{pa} values for the three monolignols are consistent with these earlier reports.

We investigated the steric factors of 3-mono and 3,5-di substituents of monolignols in more detail using synthesized analogous substances shown in Fig. 1. The oxidation rates of coniferyl (**2**), 3-ethoxy-*p*-coumaryl (**4**), 3-*n*-propoxy-*p*-coumaryl (**5**), and 3-*n*-butoxy-*p*-coumaryl alcohols (**6**) were higher than that of *p*-coumaryl alcohol (**1**) in spite of these larger substituents. These results can be explained by the order of the E_{pa} values. Among group A, an increase in the volume of the 3-substituent was found to be inversely proportional to the rate as depicted in Fig. 5B, although the E_{pa} value were similar. Thus, it was considered that some steric influences affected the order of the rates. The conformation of amino acid residues and heme at the active site of HRP are illustrated in Fig. 6. Figure 6 was made by clipping heme and amino acid residues near the heme from the structure of HRPC registered in the Protein Data Bank (ID: 7atj).[21] Furthermore, the cyanide binding to heme iron in 7atj was replaced with oxygen, the ferulic acid allocated in the active site of 7atj was replaced with sinapyl alcohol, and no geometrical optimization was conducted. From Fig. 6 and our results, the narrow entrance of HRP was likely to cause a decrease in the rates of compounds having large substituents. For 3-*t*-butoxy-*p*-coumaryl alcohol (**7**), the rate was very slow despite the fact that E_{pa} was almost the same as other alkoxyl groups. Compared with 3-*n*-butoxy-*p*-coumaryl alcohol (**6**), it is considered that the bulk of the *t*-butoxyl group decreased the reaction rate.

For 3-methyl-*p*-coumaryl alcohol (**8**), the electron-donating effect of the methyl group was slightly smaller than that of the alkoxyl group, as shown by cyclic voltammetry. However, the rate was much lower than that of group A substrates. For 3,5-dimethyl-*p*-coumaryl alcohol (**9**), which has two electron-donating groups on the phenyl ring, the rate of decrease was much slower than that of *p*-coumaryl alcohol (**1**) (Fig. 5C). The rate of 3-*n*-butoxy-*p*-coumaryl alcohol (**6**) oxidation was faster than that of *p*-coumaryl alcohol (**1**), even though the *n*-butoxy group is quite large. Furthermore, the volume of the two methoxyl groups of sinapyl alcohol (**3**) were considered to be smaller than that of the *n*-butoxyl group.[12] However, the oxidation rate of sinapyl alcohol was slower than that of 3-*n*-butoxy-*p*-coumaryl alcohol (**6**), although there was a difference in terms of flexibility of substituents. These results suggest the magnitude of the effect of steric hindrance by the disubstitution. It was also apparent from Tables 1 and 3 that the decreasing rates of group C were very slow in spite of their high electrochemical reactivity expected from E_{pa} values. Considering the relation between the reaction rates of 3-*n*-butoxy- (**6**) and 3,5-dimethyl- (**9**) *p*-coumaryl alcohols, we are of the view that the cause of the decrease in reaction rates was not merely due to the volume of the substituents but also the steric hindrance that occurred near heme iron when HRP was compound II. Sakurada et al.[22] reported on the interaction between HRP compound II and *p*-cresol using the AM-1d semiempirical molecular orbital method on the basis of the X-ray crystallographic structure. They stated that the Arg-38 residue decreased the activation energy of the reaction of HRP compound II and *p*-cresol by building a hydrogen bond with the hydroxyl oxygen of *p*-cresol. In our opinion, the amino acid residues near the heme may be reversely involved in the decrease of the rates of the 2,6-disubstituted phenols in terms of steric hindrance. Østergaad et al.[23] and Nielsen et al.[24] stated that Ile-138 and Pro-139 sterically hinder the oxidation of sinapyl alcohol (**3**) due to an overlap with the methoxyl group of sinapyl alcohol when the phenolic oxide of sinapyl alcohol forms a hydrogen bond with Arg-38. This was found by analyzing extracellular anionic peroxidase (ATP A2), which was isolated from rapidly lignifying *Arabidopsis* cell suspension culture. However, this might not be the only cause for the decreasing oxidation rate of the compounds. As described above, as the chain length of alkoxyl groups of 3-alkoxy-*p*-coumaryl alcohols increased, the rates gradually decreased. This type of hindrance was consequently considered to be due to the molecular volume of the reductants and not merely to a local part, e.g., Pro-139 and/or Leu-138 of HRP. Furthermore, comparing sinapyl alcohol (**3**) and 3,5-dimethyl-*p*-coumaryl alcohol (**9**), the latter was considered to have smaller substituents than the former in terms of an overlap of substituents with Leu-138, Pro-139 and Arg-38. In Fig. 6, the distance between the methoxyl carbon of sinapyl alcohol and the carboxyl oxygen of Pro-139 was 2.0 Å, and the distance between the methoxyl carbon and the carboxyl oxygen of Leu-138 was 2.2 Å. For 3,5-dimethyl-*p*-coumaryl alcohol (**9**), the distance between the methyl carbon of 3,5-dimethyl-*p*-coumaryl alcohol and the carboxyl carbon of Pro-139 was 2.1 Å, and the distance between the methyl carbon and the carboxyl oxygen of Leu-138 was 3.6 Å. These values were obtained by the same

method used for sinapyl alcohol (**3**). Comparing these values, it could be certainly considered that 3,5-dimethyl-*p*-coumaryl alcohol (**9**) is more loosely packed than sinapyl alcohol (**3**). However, the oxidation rate of 3,5-dimethyl-*p*-coumaryl alcohol was slower than that of sinapyl alcohol. Therefore, as mentioned above, it was considered that 3,5-dimethyl- and also 3-methyl-*p*-coumaryl alcohols (group B) preferred the condition near the entrance of HRP than the inner hydrophilic active site. We inferred from these results that when considering the effect of substituents of substrates on the oxidation of monolignols by HRP–H_2O_2 it would be necessary to take account of three factors: steric hindrance by the volume of the substrates, steric hindrance by amino acid residues near the heme, and the hydrophobic effect of substrates. To clarify these effects of amino acid residues, computational chemistry and experiments using mutations of Pro-139 and/or Arg-38 would become powerful tools.

The reactivity of lignin dimers in the oxidation by HRP has not been examined to the best of our knowledge. It is still unclear whether the steric hindrances considered in our study occur in the polymerization of lignin dimers or polymers by HRP, although from this study on dimers it was assumed that the reaction rates of dimers were much slower than corresponding monomers. If the steric hindrance that occurs in dimers and polymers is much greater than that in monolignols, it would be also interesting to know how woods overcome these steric problems and the problem of collision between lignin polymers and enzymes. Sasaki et al.[3] reported that a poplar cell wall peroxidase can oxidize the lignin polymer. Clarifying the oxidation mechanism and structure of the enzyme would help to solve the aforementioned problems.

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