Lignin dehydrogenative polymerization mechanism: a poplar cell wall peroxidase directly oxidizes polymer lignin and produces in vitro dehydrogenative polymer rich in β-*O*-4 linkage

Shinya Sasaki[a], Tomoaki Nishida[b], Yuji Tsutsumi[a,*], Ryuichiro Kondo[a]

[a] *Department of Forest and Forest Products Sciences, Kyushu University, 6-10-1 Hakozaki, Higashiku, Fukuoka 812-8581, Japan*
[b] *Department of Forest Resource Science, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan*

Received 26 January 2004; revised 20 February 2004; accepted 20 February 2004

First published online 5 March 2004

Edited by Ulf-Ingo Flügge

Abstract **An investigation was performed to determine whether lignin dehydrogenative polymerization proceeds via radical mediation or direct oxidation by peroxidases. It was found that coniferyl alcohol radical transferred quickly to sinapyl alcohol. The transfer to syringaresinol was slower, however, the transfer to polymeric lignols occurred very slightly. This result suggests that the radical mediator theory does not sufficiently explain the mechanism for dehydrogenative polymerization of lignin. A cationic cell wall peroxidase (CWPO-C) from poplar (*Populus alba* L.) callus showed a strong substrate preference for sinapyl alcohol and the sinapyl alcohol dimer, syringaresinol. Moreover, CWPO-C was capable of oxidizing high-molecular-weight sinapyl alcohol polymers and ferrocytochrome *c*. Therefore, the CWPO-C characteristics are important to produce polymer lignin. The results suggest that CWPO-C may be a peroxidase isoenzyme responsible for the lignification of plant cell walls.**

Key words: Dehydrogenative polymerization; Lignin biosynthesis; Peroxidase; Radical mediation; Direct oxidation

1. Introduction

Lignification is the process of forming the phenylpropanoid macromolecules termed lignin. Lignin is a polymeric material that is composed of phenylpropanoid units derived from three cinnamyl alcohols: *p*-coumaryl, coniferyl, and sinapyl alcohols. Lignification entails monolignol biosynthesis, transport to the cell wall, and polymerization. Lignin polymerization is thought to result from the oxidative coupling of a monolignol to the growing polymer [1–4]. Polymerization continues if a phenolic group on the lignin polymer is oxidized to a radical, either by a peroxidase (EC 1.11.1.7) or by a peroxidase-generated monolignol radical, and the phenolic radical on the polymer is coupled to a second monolignol radical.

Recently, a radical mediation model was proposed to produce a lignin molecule [5–7]. The radical mediation model postulates that the formation of monolignol radicals occurs through interaction with cell-wall-bound peroxidases and the monolignol, and newly formed radicals diffuse to the growing lignin polymer and follow one of two possible fates: (a) if the lignin polymer is at a base oxidative state, the higher oxidation state on the monolignol can be transferred to the lignin molecule, thus returning the monolignol to the ground state; (b) if the lignin polymer is at a higher oxidation state, the monolignol radical can undergo an oxidative coupling reaction to form a covalent bond. The radical mediation mechanism was suggested based on the findings that coniferyl alcohol radicals oxidize sinapyl alcohol [6,7]. Whether the monolignol radical can be transferred to the lignin macromolecule as well as to sinapyl alcohol, however, has not yet been shown. In contrast, another model proposes a direct oxidation of the growing polymer by peroxidase. The mechanism requires that peroxidases directly oxidize both lignin macromolecules and monolignols, and that a phenolic radical on the lignin macromolecule be coupled to a monolignol radical. It is unclear, however, whether peroxidase can oxidize directly three-dimensional lignin macromolecules as substrates.

Horseradish peroxidase (HRP) is a typical plant peroxidase that is used to study the mechanism of lignin dehydrogenative polymerization [8,9]. HRP oxidizes coniferyl alcohol efficiently, but is inefficient at oxidizing sinapyl alcohol. Previously, we reported that a peroxidase isoenzyme (cationic cell-wall-bound peroxidase, CWPO-C) from poplar callus preferentially uses sinapyl alcohol or syringaldazine as a substrate, unlike HRP [10–12], and that CWPO-C polymerizes sinapyl alcohol oligomers to a larger molecular weight polymer [13]. On the other hand, HRP produced little polymerization [13]. To better understand the mechanism of the formation of the dehydrogenative polymer by CWPO-C, we studied the catalytic efficiency of oxidation of dimeric and polymeric lignols by a specific peroxidase. We also studied the potential of radical transfer from the monolignol to the polymeric lignol. In this study, we demonstrate that CWPO-C is efficient at oxidizing the polymer, but a radical transfer from coniferyl alcohol to lignin polymers is incompetent.

2. Materials and methods

2.1. Plant materials and chemicals

We induced poplar (*Populus alba* L.) callus development on Murashige and Skoog basal medium supplemented with 3% sucrose, 1.0 ppm 2,4-D, 0.5 ppm kinetin, and 0.8% agar. The callus was maintained on the medium at 25°C in the dark [13]. Peroxidase isoenzyme (CWPO-C) was purified as described by Aoyama et al. [13]. HRP type VI (Sigma) was used without further purification. Peroxidase solutions were prepared (approximately 0.6–0.8 mg/ml), then the protein con-

*Corresponding author. Fax: (81)-92-642 4282.
E-mail address: y-tsutsu@agr.kyushu-u.ac.jp (Y. Tsutsumi).

0014-5793/04/$30.00
doi:10.1016/S0014-5793(04)00224-8

centration of the solution was determined. For the determination of protein content, a Bio-Rad protein assay kit was used with bovine serum albumin as the protein standard. An aliquot from the freshly prepared HRP solution or CWPO-C stock solution was used for each experiment. Sinapyl alcohol and coniferyl alcohol were synthesized by the method of Quideau and Ralph [14] and purified by silica gel column chromatography. Syringaresinol was a gift from Dr. M. Shigematsu, Gifu University, Japan. Sinapyl alcohol oligomers were prepared as described by Aoyama et al. [13]. Sinapyl alcohol polymer was prepared as described below. Ferrocytochrome *c* was prepared according to Wariishi et al. [15]. All other chemicals were purchased as extra-pure grade and used without further purification.

2.2. Preparation of sinapyl alcohol polymers

We prepared two sinapyl alcohol polymers: polymer-A and -B. Sinapyl alcohol polymer-A was prepared from sinapyl alcohol using CWPO-C. The reaction was initiated by the addition of 300 μl 500 mM H_2O_2 solution to a 5-ml mixture [40 mM Na-phosphate buffer (pH 6.8), 50 mg sinapyl alcohol in 400 μl ethanol, and 20 μg CWPO-C], and allowed to proceed for 30 min at 25°C. The resulting suspension was centrifuged at 15 000 rpm, and the water-insoluble material was collected. The material was resuspended in water and centrifuged to eliminate water-soluble materials twice more. The final material was lyophilized and subjected to gel permeation HPLC (GPC) to confirm that the acquired product was a polymer. The GPC analysis was performed using an HPLC system equipped with a photodiode array detector using an α-2500 column (Toso, Japan). A dioxane–water mixture (4:1, v/v) containing 40 mM LiCl was used as the eluent, and the flow rate was 0.5 ml/min [13]. For the experiments of the direct oxidation of lignin polymers by peroxidase, polymer-A was purified by GPC to eliminate oligomers. As for the isolation of the polymer, the same analytical conditions were used as mentioned above, with the exception that 40 mM LiCl was omitted from the eluent. The polymer-A preparation was resolved into four fractions [I: polymer fraction (retention time 11.2–12.0 min), II: oligomer fraction (retention time 12.0–13.5 min), III: dimer fraction (retention time 13.5–15.0 min), IV: monomer fraction (retention time 15.0–16.5 min)]. The obtained polymer fraction (I) was concentrated by evaporation, then lyophilized. Finally, we confirmed that the polymer fraction did not contain any low molecular weight materials (oligomers, dimers, and monomers) by the above mentioned GPC system.

Sinapyl alcohol polymer-B was prepared from sinapyl alcohol oligomer and sinapyl alcohol using CWPO-C. The reaction was initiated by the addition of 600 μl of 500 mM H_2O_2 solution to a 10-ml mixture [40 mM Na-phosphate buffer (pH 6.8), 20 mg sinapyl alcohol in 500 μl ethanol, 20 mg sinapyl alcohol oligomer in 625 μl dioxane–H_2O solution (4:1 v/v) and 20 μg CWPO-C]. The reaction proceeded for 30 min at 25°C. The resulting suspension was centrifuged at 15 000 rpm, and the water-insoluble material was collected. The material was resuspended in water and centrifuged to eliminate water-soluble materials twice more. The final material was lyophilized and subjected to GPC to confirm that the obtained product was a polymer as described above.

2.3. Peroxidase reaction with coniferyl alcohol as a radical mediator

The reaction mixture (3 ml) contained HRP, 0.1 mM coniferyl alcohol and 50 μM H_2O_2 in 40 mM phosphate buffer (pH 6.8). An equivalent amount of sinapyl alcohol (0.1 mM), syringaresinol (0.1 mM) or sinapyl alcohol polymer-A (same weight as sinapyl alcohol) was also added. To facilitate the solubilization of the substrates, the reaction mixture also contained 4% dioxane and 0.15% ethanol in the final concentrations. The reaction was initiated by adding H_2O_2 and was carried out at 30°C. Aliquots of the reaction mixture were sampled sequentially. Reactions were terminated by adding a half volume of 36% acetonitrile aqueous solution containing 6% trifluoroacetic acid, and each sample was then frozen in liquid nitrogen. The samples were subjected to HPLC analysis [column: Wakosil II 5C18G column (4.6×250 mm); for sinapyl alcohol reaction, eluent: 20 mM phosphate buffer (pH 3.0)/acetonitrile (88:12, v/v), flow rate: 1.0 ml/min, and detection: 272 nm; for syringaresinol reaction, eluent: 20 mM phosphate buffer (pH 3.0)/methanol (50:50, v/v), flow rate: 0.7 ml/min, and detection: 280 nm] to determine the consumption of coniferyl alcohol and sinapyl alcohol.

Sinapyl alcohol and syringaresinol oxidizing activity of HRP and CWPO-C were also determined. Each peroxidase reaction was carried out without coniferyl alcohol. The analytical procedure was the same as above.

2.4. Peroxidase oxidation of polymeric substrates

Both peroxidases (CWPO-C, 3.2 μM; HRP, 2.8 μM) were converted to their oxidized states by adding an equivalent amount of H_2O_2 to 50 μl of 40 mM phosphate buffer (pH 6.8) in a cuvette. After the conversion of peroxidase to its oxidized state was confirmed, sinapyl alcohol polymer-A was added to the reaction mixture. The reaction was carried out at 15°C for 1 min in a spectrophotometer (Shimadzu UV-230) equipped with a thermo-controller. The UV-Vis spectral change, which is dependent on the reduction of peroxidase, was recorded.

In the oxidation of ferrocytochrome *c* by peroxidase, the reaction mixture (1 ml) contained 40 μM ferrocytochrome *c*, 10 μg of CWPO-C or 10 μg of HRP, and 50 μM H_2O_2 in 40 mM phosphate buffer (pH 6.8). The reaction was initiated by adding H_2O_2 to the mixture and was carried out at 30°C. The absorbance decrease at 550 nm (depending on the oxidation of ferrocytochrome *c*) was monitored at 30°C.

2.5. Thioacidolysis of sinapyl alcohol polymer prepared by CWPO-C

Thioacidolysis of sinapyl alcohol polymer-A, -B, and sinapyl alcohol oligomer and subsequent GC-MS analyses were performed as described by Hamada et al. [16].

The degradation product, 1-syringyl-1,2,3-trithioethylpropane (S-T), was determined by GC-MS (Shimadzu) analysis as described by Hamada et al. [16].

3. Results

3.1. Monomer radicals do not transfer to polymeric lignols

Sinapyl alcohol is not a favored substrate for the majority of general plant peroxidases such as HRP. However, peroxidases can oxidize this substrate through a radical transfer from hydroxycinnamic acids and hydroxycinnamyl alcohols. If coniferyl alcohol is the radical mediator of the oxidation of sinapyl alcohol, the coniferyl alcohol radical should revert to its ground state, and sinapyl alcohol should be oxidized. Thus, at first we investigated the oxidation of the mixture of sinapyl alcohol and coniferyl alcohol (Fig. 1).

As shown in Fig. 1, coniferyl alcohol was decreased, when it was used as a single substrate for HRP. In the oxidation of the mixture of coniferyl alcohol and sinapyl alcohol, however, coniferyl alcohol levels decreased very slightly. Conversely, the oxidation of sinapyl alcohol by HRP resulted in a faster decrease of sinapyl alcohol levels in the presence of coniferyl alcohol. When sinapyl alcohol was almost entirely consumed, coniferyl alcohol levels began to decrease. As a result, syringaresinol was a main product in the coniferyl-alcohol-mediated reaction. These profiles indicate that the coniferyl alcohol radical generated by HRP transfers to sinapyl alcohol quickly, and confirm the previously proposed radical mediation mechanism [6,7].

Coupling of the growing polymer and monolignol is essential in the dehydrogenative polymerization of lignin. Therefore, we looked at monolignol radicals and whether they transfer to lignin dimers and polymers as well as to monomers.

As a result of the oxidation in the mixture of coniferyl alcohol and syringaresinol by HRP, there was a faster decrease of coniferyl alcohol than in the mixture with sinapyl alcohol (Fig. 2). In other words, there was a slower transfer of the coniferyl alcohol radicals to syringaresinol as compared with sinapyl alcohol. Coniferyl alcohol levels decreased at the same quick rate, whether the HRP oxidative substrate was only coniferyl alcohol or a mixture of coniferyl alcohol and

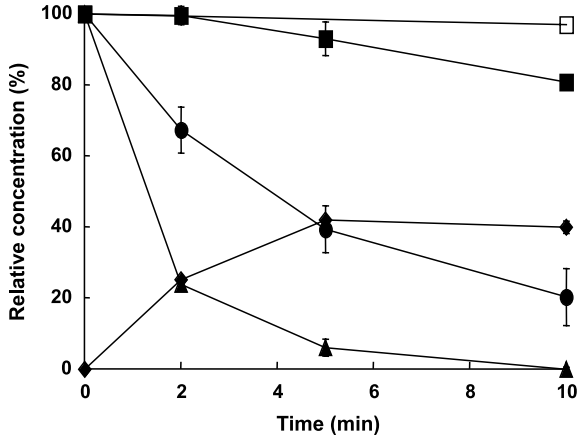

Fig. 1. HRP-mediated reaction of the mixture of coniferyl and sinapyl alcohol. A mixture containing 0.1 mM coniferyl alcohol and 0.1 mM sinapyl alcohol was treated with HRP in the presence of 50 μM H_2O_2. Aliquots of the reaction mixture were sampled sequentially. Samples were analyzed by HPLC to determine the consumption of coniferyl alcohol and sinapyl alcohol. Data shown are the means of triplicate analyses ± S.D. ●, decrease in coniferyl alcohol as a single substrate (control experiment); □, decrease in sinapyl alcohol as a single substrate (second control experiment); ▲, decrease in sinapyl alcohol; ■, decrease in coniferyl alcohol; ♦, production of syringaresinol.

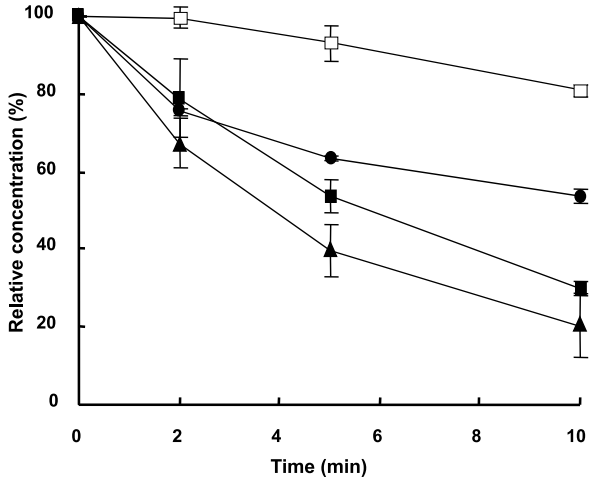

Fig. 2. Consumption of coniferyl alcohol in the presence of various syringyl derivatives in HRP reactions. A mixture of 0.1 mM coniferyl alcohol and 0.1 mM sinapyl alcohol, syringaresinol, or sinapyl alcohol polymers (0.1 mM C_6–C_3 units) was treated with HRP in the presence of 50 μM H_2O_2. Aliquots of the reaction mixtures were sampled sequentially. Samples were analyzed by HPLC to determine coniferyl alcohol consumption. □, addition of sinapyl alcohol; ●, addition of syringaresinol; ■, addition of sinapyl alcohol polymer; ▲, no addition (control experiment).

sinapyl alcohol polymers (Fig. 2). This result implies that coniferyl alcohol radicals do not transfer to sinapyl alcohol polymers, but rather the coniferyl alcohol radicals immediately coupled each other.

3.2. Direct oxidation of polymeric lignol by CWPO-C

Observing the oxidation of sinapyl alcohol and dimeric syringaresinol by HRP, the oxidation rate of the dimer was seen to be nine-fold lower than that of the monomer (Table 1). Correspondingly the oxidation by CWPO-C retained a high oxidative capacity toward syringaresinol as compared with HRP (Table 1).

In the following experiments, we investigated whether CWPO-C oxidizes sinapyl alcohol polymers. Addition of the equivalent amount of H_2O_2 converted CWPO-C to its oxidized state (a mixture of compounds I and II), and spectral changes were determined after the addition of sinapyl alcohol polymers. Fig. 3 shows that oxidized CWPO-C was reduced to its native state by the addition of the sinapyl alcohol polymers, indicating that CWPO-C oxidized the polymer. In contrast, the oxidized state of HRP was absolutely unchanged by polymeric substrate addition (data not shown).

The oxidation of ferrocytochrome *c* by CWPO-C or HRP was also investigated. The molecular mass of ferrocytochrome *c* is quite large, therefore the heme pocket of peroxidases cannot oxidize ferrocytochrome *c* [15]. As can be seen in Fig. 4, the obvious decrease of the absorbance in the CWPO-C-mediated reaction indicates that CWPO-C oxidizes

Table 1
Oxidation of sinapyl alcohol and syringaresinol by peroxidases

	Sinapyl alcohol	Syringaresinol
CWPO-C	962 ± 66	1266 ± 52
HRP	98 ± 3	11 ± 1

Oxidation rate was expressed as consumed substrate in μmol/min/mg protein. Date are means of triplicate analyses ± S.D.

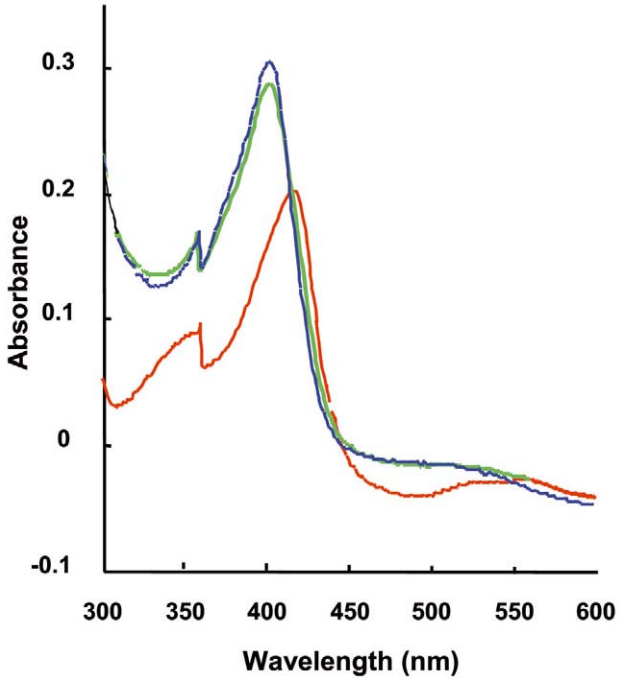

Fig. 3. The reaction of CWPO-C compounds I and II by the addition of sinapyl alcohol polymers. 3.2 μM CWPO-C was converted to its oxidized state (red line, a mixture of compounds I and II) by adding an equivalent amount of H_2O_2 to 50 μl of CWPO-C solution in 40 mM phosphate buffer (pH 6.8) in a cuvette. Sinapyl alcohol polymers were added to the enzyme solution. UV-Vis spectral changes were recorded to determine the reduction of the peroxidase. Blue line, native-state CWPO-C; red line, oxidized-state CWPO-C (a mixture of compounds I and II); green line, following the addition of sinapyl alcohol polymers.

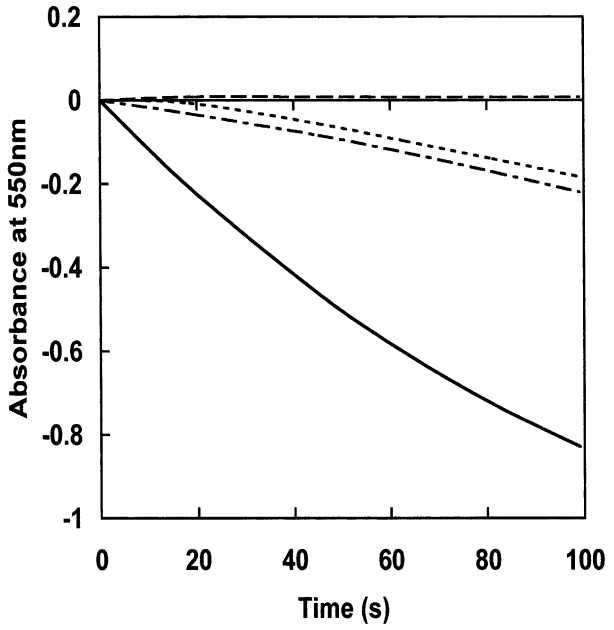

Fig. 4. Oxidation of ferrocytochrome *c* by CWPO-C or HRP. The reaction mixture (1 ml) contained 40 μM ferrocytochrome *c*, 10 μg of CWPO-C or 10 μg of HRP, and 50 μM H_2O_2 in 40 mM phosphate buffer (pH 6.8). The reaction was initiated by adding H_2O_2 to the mixture and was carried out at 30°C. The decrease in absorbance at 550 nm, depending on the oxidation of ferrocytochrome *c*, was monitored. Dashed line, spontaneous oxidation; dotted line, H_2O_2; dash-dotted line, HRP and H_2O_2; solid line, CWPO-C and H_2O_2.

ferrocytochrome *c* quickly, whereas HRP does not. This fact thus contributes to the evidence that CWPO-C is capable of oxidizing high-molecular-weight substrates such as polymers.

3.3. *Synthetic lignin produced by CWPO-C contains more β-O-4 linkages*

As Sarkanen stated, so-called end-wise polymers containing β-*O*-4 linkages at high frequency can be formed by coupling individual monolignols to a growing polymer [1]. As shown in the above experiment, HRP does not oxidize polymeric lignols; therefore, HRP may not produce polymers containing a high proportion of β-*O*-4 linkages. In contrast, CWPO-C is expected to produce polymers rich in β-*O*-4 linkages.

We performed a thioacidolysis of the water-insoluble dehydrogenative products prepared by CWPO-C or HRP, and the yield of the degradation product (S-T) was compared. The S-T yield from sinapyl alcohol oligomers produced from HRP was 51.6 ± 7.9 μmol/g (Table 2). Polymers produced by CWPO-C in reactions containing sinapyl alcohol oligomers contained much more β-*O*-4 linkages. This indicates that the coupling of monomers to oligomers proceeds via β-*O*-4 at a high frequency. When the CWPO-C-mediated polymerization reactions were initiated from sinapyl alcohol, the S-T yield from the polymers increased further to 278 ± 18 μmol/g (Table 2). CWPO-C produces polymers containing a high proportion of β-*O*-4 linkages. This feature can be attributed to the fact that CWPO-C oxidizes high-molecular-weight substrates, including polymeric lignols.

4. Discussion

In our previous paper, a reaction mixture containing a 1:1 ratio (w/w) of sinapyl alcohol oligomers and sinapyl alcohol was oxidized by either CWPO-C or HRP, and the molecular weight distributions of the reaction products were determined by gel permeation chromatography. In the HRP reaction, high-molecular-weight polymers were rarely formed; instead, sinapyl alcohol was predominantly converted to oligomers and dimeric syringaresinol [13].

In this paper, we show that oxidation of syringaresinol by HRP is markedly slower than that of sinapyl alcohol, and that HRP is almost unable to oxidize sinapyl alcohol polymers. Thus, the order of preference for HRP oxidation substrates is: monomer > dimer ≫ polymer. We therefore predict that radical coupling of sinapyl alcohol oligomers with sinapyl alcohol is rare, whereas the coupling of monomer radicals is dominant. In contrast, CWPO-C oxidizes syringaresinol, a sinapyl alcohol dimer, as well as sinapyl alcohol. Furthermore, the results in Figs. 3 and 4 show that CWPO-C is capable of oxidizing high-molecular-weight lignin polymers. Thus, in the CWPO-C-mediated reaction, radical coupling occurs between monolignol and polymeric lignols, resulting in growing lignin polymers of high molecular weight.

Lignin polymers can be formed by the coupling of individual monolignols to a growing polymer [1]. This end-wise polymerization mechanism explains why β-*O*-4 linkages are the most abundant linkage type in lignin. If CWPO-C is more efficient than HRP in oxidizing a growing polymer, β-*O*-4 linkages would be formed at a high frequency in CWPO-C-mediated polymerization. Thioacidolysis revealed that the dehydrogenated polymer produced by CWPO-C did contain a larger proportion of β-*O*-4 linkages than did the HRP product.

Slow addition of the monolignol to the polymerization reactions causes a greater increase in the proportion of β-*O*-4 linkages in dehydrogenated polymer [9]. HRP oxidizes growing polymers (probably oligomers) rather slowly, but oxidizes monomers quickly, so that the slow addition of the monolignol is essential to oxidize the lignin oligomers. It is striking that CWPO-C produces polymeric lignols containing a large proportion of β-*O*-4 linkages even in the 'Zulaufverfahren' condition. This is clearly due to the ability of CWPO-C to oxidize polymeric lignols, whereas the fact that fewer β-*O*-4 linkages are produced by HRP is due to the poor oxidizing ability of HRP toward polymeric lignols. This ability of

Table 2
Yield of syringyl monomer (S-T) from synthetic lignin polymer by thioacidolysis

	Enzyme	Substrate	S-T yield (μmol/g) (lignin)
Sinapyl alcohol polymer-A	CWPO-C	sinapyl alcohol	278 ± 18
Sinapyl alcohol polymer-B	CWPO-C	sinapyl alcohol+sinapyl alcohol oligomer (1:1)	165 ± 24
Sinapyl alcohol oligomer	HRP	sinapyl alcohol	51.6 ± 7.9

S-T, 1-syringyl-1,2,3-trithioethylpropane. Data are means of triplicate analyses ± S.D.

CWPO-C seems to be essential for the formation of β-*O*-4-rich lignin in cell walls.

In this study, we examined whether lignin dehydrogenative polymerization proceeds by radical mediation from monolignols. Radical transfer is a non-enzymatic reaction, and the rate of radical transfer depends strongly on the oxidation potential of the substrates. The oxidation potential of sinapyl alcohol is estimated to be 0.727 V, which is lower than that of coniferyl alcohol (0.810 V). Therefore, coniferyl alcohol radicals produced by the action of HRP transfer easily to sinapyl alcohol. However, the oxidation potentials of syringaresinol (0.903 V) and the β-*O*-4 syringyl dimer (0.866 V) are larger than that of coniferyl alcohol (0.810 V) (oxidation potentials are the unpublished data of Dr. Shigematsu).

In our experiments, coniferyl alcohol was consumed more rapidly in the presence of syringaresinol than in the presence of sinapyl alcohol, suggesting that radical transfer is much slower from coniferyl alcohol to syringaresinol than to sinapyl alcohol. Coniferyl alcohol was consumed most rapidly in the presence of sinapyl alcohol polymers. In this experiment, we should be concerned about the fact that the polymeric substrate carries fewer epitopes for the oxidation than the monomeric substrate when the polymeric substrate was used for the same number of C6–C3 units as the monomer substrate. However, we previously showed that HRP did not catalyze the polymerization of a mixture of sinapyl alcohol oligomers and sinapyl alcohol with coniferyl alcohol as the radical mediator, but this reaction system largely produced sinapyl alcohol dimer and oligomer [13]. These results in Fig. 2 and a previous study suggest that the coniferyl alcohol radical rarely transfers to polymeric lignols and thus cannot mediate the dehydrogenative polymerization of lignin.

Unlike other peroxidases, CWPO-C prefers sinapyl alcohol rather than coniferyl alcohol as a substrate [17]. CWPO-C catalyzes the oxidation of three-dimensional lignin polymers and ferrocytochrome *c*. Ferrocytochrome *c* is a 13-kDa heme protein previously used as a polymeric model compound during oxidative degradation by fungal lignin peroxidase (LiP) [15]. LiP oxidizes ferrocytochrome *c* directly, and the Trp171 moiety on the LiP surface has been postulated to be the active site [18–20]. Recently, it was reported that sinapyl alcohol does not fit within the substrate binding site of HRP C and *Arabidopsis* ATP A2 peroxidase [21]. These studies showed that coniferyl alcohol and *p*-coumaryl alcohol fit, but sinapyl alcohol does not. Similar fits were predicted to be general for all plant peroxidases [22]. Thus, CWPO-C is expected to have a site for the oxidation of lignin polymers at its surface, similar to LiP and cytochrome *c* peroxidase [23,24].

Acknowledgements: We are grateful to Associate Professor M. Shigematsu, Gifu University, for his kind gift of authentic syringaresinol and useful discussion on the oxidation potentials for monomeric and dimeric lignols. This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan (15380121).

References

[1] Sarkanen, K.V. (1971) Lignins, pp. 95–163, Wiley-Interscience, New York.
[2] Harkin, J.M. (1967) in: Oxidative Coupling of Phenols (Taylor, W.I. and Battersby, A.R., Eds.), pp. 243–321, Marcel Dekker, New York.
[3] Freudenberg, K. and Neish, A.C. (1968) Constitution and Biosynthesis of Lignin, Springer-Verlag, Berlin.
[4] Adler, E. (1977) Wood Sci. Technol. 11, 169–218.
[5] Ronald, H. and Wilfred, V. (2001) Plant Physiol. 126, 1351–1357.
[6] Takahama, U. (1995) Physiol. Plant. 93, 61–68.
[7] Takahama, U. and Oniki, T. (1997) Plant Cell Physiol. 38, 456–462.
[8] Syrjnen, K. and Brnow, G. (1998) J. Chem. Soc. Perkin Trans. 1, 3425–3429.
[9] Syrjnen, K. and Brnow, G. (2000) J. Chem. Soc. Perkin Trans. 1, 183–187.
[10] Tsutsumi, Y. and Sakai, K. (1994) Mokuzai Gakkaishi 40, 744–750.
[11] Tsutsumi, Y., Nishida, T. and Sakai, K. (1994) Mokuzai Gakkaishi 40, 1348–1354.
[12] Tsutsumi, Y., Matsui, K. and Sakai, K. (1998) Holzforschung 52, 275–281.
[13] Aoyama, W., Sasaki, S., Matsumura, S., Hirai, H., Tsutsumi, Y. and Nishida, T. (2002) J. Wood Sci. 48, 497–504.
[14] Quideau, S. and Ralph, J. (1992) J. Agric. Food Chem. 40, 1108–1110.
[15] Wariishi, H., Sheng, D. and Gold, M.H. (1994) Biochemistry 33, 5545–5552.
[16] Hamada, K., Tsutsumi, Y., Yamauchi, K., Fukushima, K. and Nishida, T. (2004) J. Wood Sci. 49, 333–338.
[17] Matsui, K., Tsutsumi, Y. and Sakai, K. (1997) Mokuzai Gakkaishi 43, 778–784.
[18] Blodig, W., Smith, A.T., Doyle, W.A. and Piontek, K. (2001) J. Mol. Biol. 26, 851–861.
[19] Sollewijn Gelpke, M.D., Lee, J. and Gold, M.H. (2002) Biochemistry 41, 3498–3506.
[20] Choinowski, T., Blodig, W., Winterhalter, K.H. and Piontek, K. (1999) J. Mol. Biol. 286, 809–827.
[21] Ostergaard, L., Teilum, K., Mirza, O., Mattsson, O., Petersen, M., Welinder, K.G., Mundy, J., Gajhede, M. and Henriksen, A. (2000) Plant Mol. Biol. 44, 231–243.
[22] Nielsen, K.L., Indiani, C., Henriksen, A., Feis, A., Becucci, M., Gajhede, M., Smulevich, G. and Welinder, K.G. (2001) Biochemistry 40, 11013–11021.
[23] Johjima, T., Itoh, N., Kabuto, M., Tokimura, F., Nakagawa, T., Warishi, H. and Tanaka, H. (1999) Proc. Natl. Acad. Sci. USA 96, 1989–1994.
[24] Miller, MA., Vitello, L. and Erman, J.E. (1995) Biochemistry 34, 12048–12058.