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Sinapyl alcohol-specific peroxidase isoenzyme catalyzes the formation of the dehydrogenative polymer from sinapyl alcohol

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Abstract Two peroxidases, CWPO-A and CWPO-C, were isolated from the cell walls of poplar (*Populus alba* L.) callus culture. The cationic CWPO-C showed a strong preference for sinapyl alcohol over coniferyl alcohol as substrate. Thus, the monolignol utilization of CWPO-C is unique compared with other peroxidases, including anionic CWPO-A and horseradish peroxidase (HRP). CWPO-C polymerized oligomeric sinapyl alcohol (S-oligo) and sinapyl alcohol, producing a polymer of greater molecular weight. In contrast, HRP, which is specific to coniferyl alcohol, produced sinapyl alcohol dimers, rather than catalyzing polymerization. Adding coniferyl alcohol as a radical mediator in the HRP-mediated reaction did not result in S-oligo polymerization. This report shows that CWPO-C is an isoenzyme specific to sinapyl alcohol that polymerizes oligomeric lignols. Its catalytic activity toward oligomeric lignols may be related to the lignification of angiosperm woody plant cell walls.

Key words Dehydrogenative polymerization · Lignin biosynthesis · Peroxidase · Substrate utilization · Sinapyl alcohol

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Introduction

In plant taxa, peroxidases (EC 1.11.1.7) are ubiquitous and thought to participate in a wide range of physiologic functions, such as lignification,[1–3] resistance to pathogens,[4,5] and catabolism of plant hormones such as auxins.[6] A number of peroxidase isoenzymes have been recognized in plant cells, and the functions of several have been determined.

The last step of lignin biosynthesis in vascular plants, oxidative polymerization of monolignols, is catalyzed by peroxidases or laccase.[1] Researchers have investigated whether some specific peroxidase isoenzymes are correlated with lignification in many plants, especially tobacco[7,8] and poplar.[9–12] In these studies peroxidase isoenzymes are often split into three subgroups based on their isoelectrophoretic points: anionic, neutral, and basic. Mäder et al. suggested that acidic (anionic) peroxidases are responsible for lignification in tobacco based on their observation that an anionic isoenzyme was highly active during polymerization of coniferyl alcohol and *p*-coumaryl alcohol.[13] Later studies suggested that anionic peroxidase is the key isoenzyme for lignification in poplar.[10–12] In contrast, other studies implicated wall-bound cationic peroxidase isoenzymes in lignification.[14,15] Methods have now been developed that use transgenic plants that over- or underexpress specific (acidic) peroxidase genes.[16] However, this approach has failed to determine whether acidic peroxidases and lignification are correlated,[16] and the in vivo function of specific peroxidases in lignification remains unknown. In addition, researchers have studied the localization and oxidizing activity of syringaldazine by acidic or basic peroxidases as a possible correlate with lignification.[10–12,17] Syringaldazine is a substrate commonly used to detect lignification-specific peroxidases, as Harkin and Obst[18] used it to stain lignifying xylem cells. Syringaldazine shares its pattern of substitution on an aromatic ring of a hydroxyl group and two methoxyl groups with sinapyl alcohol, a monolignol of syringyl lignin. However, as pointed out by Lewis and Yamamoto, studies have not obtained definite evidence for the substitution of monolignols by syringaldazine and have rarely used

monolignols, actual lignin precursors, to investigate the role of peroxidase isoenzyme in lignin biosynthesis.[3]

Gymnosperms contain guaiacyl lignin almost solely, whereas the lignin of angiosperms is composed of both syringyl lignin and guaiacyl lignin. As shown in white birch (*Betula papyrifera*),[19] lignin in the secondary cell wall of fiber cells is mainly composed of syringyl units, whereas lignin in the middle lamellae and in vessel cell walls consists primarily of guaiacyl units in typical angiosperm woody plants. The process by which lignin is biosynthesized regulates this heterogeneous deposition of syringyl and guaiacyl lignin in angiosperms. Previously, we reported that the peroxidase fraction ionically bound to cell walls (CWPO) in poplar preferentially uses sinapyl alcohol or syringaldazine as substrate, unlike horseradish peroxidase (HRP) or other peroxidases present in poplar callus.[20,21] Furthermore, the amount of CWPO required to polymerize sinapyl alcohol is an order of magnitude less than for the cytoplasmic peroxidase fraction or other peroxidases.[20,21] It was recently reported that peroxidase isoenzymes with low substrate preference for sinapyl alcohol can oxidize the compound in the presence of phenolic compounds, such as hydroxycinnamic acids and coniferyl alcohol.[22,23] Phenolic compound radicals generated by peroxidase may be radical mediators that can oxidize sinapyl alcohol. Researchers have traced monolignol consumption in reaction mixtures spectrophotometrically to determine the oxidation rate of monolignol by peroxidase isoenzymes, but they did not monitor monolignol polymerization.[22,23] Furthermore, few studies have attempted to correlate specific peroxidase isoenzymes with lignification by investigating their ability to catalyze the polymerization of monolignols.[13,20,21]

In this study, we demonstrated that a purified wall-bound peroxidase isoenzyme, CWPO-C, which strongly prefers sinapyl alcohol as a substrate, catalyzes in vitro polymerization of sinapyl alcohol to form a syringyl polymer.

Materials and methods

Plant materials and chemicals

We induced poplar (*Populus alba* L.) callus to develop on Murashige and Skoog basal medium supplemented with 3% sucrose, 2,4-dichlorophenoxyacetic acid 1.0 ppm, kinetin 0.5 ppm, and 0.8% agar. The callus was maintained on the medium at 25°C in the dark.[24] Sinapyl alcohol and coniferyl alcohol were synthesized by the method described by Quideau and Ralph[25] and purified by silica gel column chromatography. Sinapyl alcohol oligomer (S-oligo) was prepared using HRP to oxidize sinapyl alcohol, as described below. All other chemicals, extra pure grade, were purchased and used without further purification, except for ferulic acid and sinapic acid. These cinnamic acid derivatives were purified before use by recrystallization in a mixture of hot methanol and water.

Preparation of S-oligo

S-oligo was prepared from sinapyl alcohol using HRP. The reaction was initiated by adding 1.5 ml H_2O_2 solution (800 mM) to a 15-ml mixture (40 mM Na phosphate buffer pH 6.8, 155 mg sinapyl alcohol, 140 μg HRP type VI (Sigma)) and proceeded with stirring for 15 min at 25°C. The resulting suspension was centrifuged at 15000 rpm, and the water-insoluble material was collected. This material was resuspended in water and centrifuged to eliminate water-soluble materials two more times. The final material was lyophilized and subjected to gel-permeation high-performance liquid chromatography (GPC) (see Fig. 4).

Purification of peroxidase isoenzymes from poplar callus cell walls

Poplar callus was subcultured, grown for 4 weeks, and harvested. Crude wall-bound peroxidase from callus cell walls was prepared according to the method described in our previous paper.[24] The homogenized poplar callus was extracted three times with 50 mM Tris-HCl buffer (pH 7.5) to ensure complete extraction of the soluble (cytoplasmic) peroxidases. The cell wall residue was incubated with the same buffer plus 0.6 M NaCl to extract the peroxidases bound ionically to the cell walls (ionically bound peroxidase, CWPO). The CWPO was concentrated via ultrafiltration (cutoff <10000; Advantec) and purified as follows.

Two cell wall peroxidase isoenzymes were isolated from the crude CWPO according to our methods[26] except that a HiPrep 16/10 Butyl column (Amersham Pharmacia) and HiLoad 16/60 Superdex 75 prep grade (Amersham Pharmacia) were used for hydrophobic chromatography and gel-filtration chromatography, respectively. The finally obtained guaiacyl-specific and syringyl-specific peroxidases were designated CWPO-A and CWPO-C, respectively. The protein content of the purified sample was determined using a Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard.

Peroxidase assay

Peroxidase was assayed by monitoring the consumption of coniferyl and sinapyl alcohols. The reaction mixture (3 ml) contained the purified peroxidase, 0.1 mM monolignol, and 50 μM H_2O_2 in 40 mM phosphate buffer (pH 6.8). The reaction, initiated by adding H_2O_2 to the reaction mixture, was carried out at 30°C. At the scheduled reaction time (2 min), we recorded the absorbance at 263 and 272 nm for coniferyl alcohol and sinapyl alcohol, respectively. Differences in absorbance were converted to the amount of consumed monolignol using extinction coefficients of 15100 and 14100 l/mol·cm for coniferyl and sinapyl alcohol, respectively. The activity during peroxidase purification was determined using guaiacol and syringaldazine as substrates. In this assay system, increases in absorbance due to the peroxidase reaction were monitored at 470 and 530 nm for guaiacol and syringaldazine, respectively.

To determine the degree of sinapyl alcohol oxidization in the presence of cinnamic acids or coniferyl alcohol, we used 3 ml of a reaction mixture containing peroxidase, 0.1 mM sinapyl alcohol, several concentrations of cinnamic acids or coniferyl alcohol, 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 reaction mixture and was carried out at 30°C. In mixtures containing cinnamic acids, sinapyl alcohol oxidation was monitored at the isosbestic point of each cinnamic acid (250 nm for ferulic acid and 258 nm for sinapic acid). In mixtures containing coniferyl alcohol, sinapyl alcohol oxidation was monitored at 280 nm, the isosbestic point for coniferyl alcohol. To calculate the rate of sinapyl alcohol oxidation, we used the following extinction coefficients of sinapyl alcohol (for the different isosbestic points): 7150 l/mol·cm (250 nm), 10140 l/mol·cm (258 nm), and 12500 l/mol·cm (280 nm).

The consumption of sinapyl alcohol and coniferyl alcohol was also determined by high-performance liquid chromatography (HPLC). The reactions were carried out as described above, except that 0.15 mM of each sinapyl alcohol and coniferyl alcohol was used. Aliquots of the reaction mixture were sequentially sampled. The reactions were terminated by adding a half volume of 36% acetonitrile aqueous solution containing 6% trifluoroacetic acid to each sample; the sample was then subjected to HPLC analysis [column: Wakosil II 5C18 HG (4.6 × 250 mm); eluent: 10 mM phosphate buffer (pH 3.0), acetonitrile (88:12 v/v); flow rate 1.0 ml/min; detection 267 nm].

Native PAGE analyses

Native polyacroylamide gel electrophoresis (PAGE) for anionic protein was performed according to our method previously described.[24] Briefly, Tris-HCl buffer (pH 8.8) and Tris-glycine were used for the gel and electrodes buffer. Native PAGE for cationic protein used 0.6 M HEPES buffer (pH 5.0) for the gel, 113 mM 6-aminohexanoic acid solution (pH 5.3) for the anodic buffer, and aqueous acetic acid (pH 3.1) for the cathodic buffer, as outlined in the Amersham Pharmacia manual. After native PAGE, bands were visualized by incubating the gel in 50 mM Tris-HCl (pH 7.5) in the presence of H_2O_2 and 2,6-dimethoxyphenol or guaiacol for 10 min at 30°C.

Dehydrogenative polymerization of S-oligo and sinapyl alcohol by peroxidases

The reaction mixture (1 ml) consisted of 40 mM phosphate buffer (pH 6.8), 0.8 mg S-oligo, 0.8 mg sinapyl alcohol, peroxidase, and 20 mM H_2O_2. The mixture also contained 4% dioxane and 5% ethanol to dissolve S-oligo and sinapyl alcohol in the reaction mixture. To investigate the effect of coniferyl alcohol on sinapyl alcohol polymerization, we added 0.08 or 0.04 mg of coniferyl alcohol to the reaction mixture. The reaction was initiated by adding H_2O_2 and was carried out at 30°C for an hour. The water-insoluble dehydrogenative polymer (DHP) formed after the reaction; it was recovered by centrifugation and washed by resuspension in water and centrifugation. The pellet was then dissolved in 1 ml dioxane and water (4:1 v/v) containing 40 mM LiCl; and 20 μl of the sample was analyzed by GPC. When the whole reaction mixture was subjected to GPC analysis, 4 ml dioxane was added to the mixture, and 100 μl of the sample was analyzed by GPC.

The DHP was analyzed by GPC using an HPLC system equipped with a photodiode-array detector with 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.

Results

Two peroxidase isoenzymes isolated from poplar callus cell wall

Denaturing sodium dodecyl sulfate (SDS)-PAGE suggested that the peroxidase preparations we used were purified to homology (data not shown). As previously reported, purified CWPO-C exhibited activity that was highly specific to syringaldazine. In contrast, the other isoenzyme, CWPO-A, exhibited high specificity for guaiacol but not for syringaldazine. The isolated peroxidase isoenzymes were subjected to anionic or cationic native PAGE, and the gels were stained with guaiacol or 2,6-dimethoxyphenol in the presence of H_2O_2 (Fig. 1). The PAGE followed by active

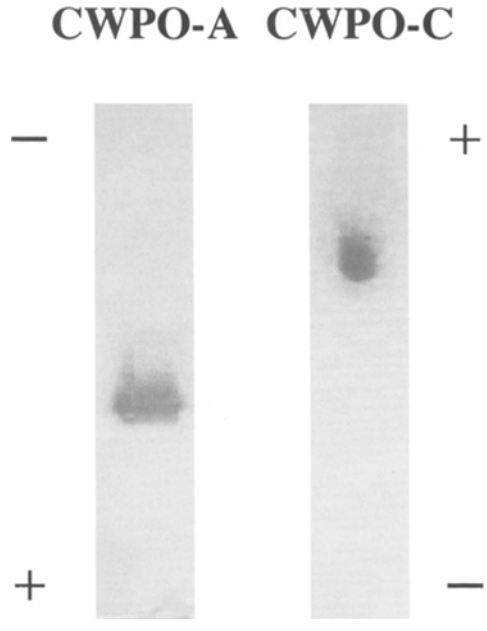

Fig. 1. Native polyacrylamide gel electrophoresis (PAGE) of the anionic CWPO-A and the cationic CWPO-C. CWPO-A was separated by anionic PAGE (pH 8.8) on a 10% polyacrylamide gel and stained with guaiacol. CWPO-C was separated by cationic PAGE (pH 5.0) on a 10% polyacrylamide gel and stained with 2,6-dimethoxyphenol. In both cases, staining was carried out in Tris-HCl buffer (pH 7.5) with each substrate and H_2O_2 for 10 min at 30°C

staining showed that CWPO-C migrated toward the cathode in the HEPES-buffered gel and vice versa for CWPO-A in Tris-buffered gel (Fig. 1). Thus, the result clearly indicates that CWPO-A and CWPO-C are the anionic and cationic isoenzymes, respectively. The cationic peroxidase, CWPO-C, stained much better with 2,6-dimethoxyphenol than with guaiacol, and the opposite was true for CWPO-A, the anionic peroxidase.

The RZ value (Reinheitszhal, the A_{405}/A_{280} absorbance ratio) of CWPO-C was approximately 2.3. Its RZ value is lower than that of other peroxidases, which is typical for this isoenzyme. The fraction containing CWPO-C was isolated using hydrophobic chromatography (HiPrep Butyl) with an NaCl gradient of 2.0–0.5 M. Peroxidase isoenzymes other than CWPO-C eluted without binding to the column. CWPO-C bound to the column at both the initial and reduced NaCl concentrations (2.0 M and ~1.3 M, respectively), suggesting that this isoenzyme is a hydrophobic protein. This hypothesis was also supported by the fact that CWPO-C was precipitated mainly by salting samples with 30%–50% of the amount of $(NH_4)_2SO_4$ required for saturation (data not shown). CWPO-C has a lower RZ value and is hydrophobic, probably due to the fact that it contains more aromatic amino acids than other peroxidases. CWPO-C was less stable than CWPO-A in buffers of low ionic strength, which may be due to self-co-aggregation. This feature made determination of the pI value by isoelectric focusing (IEF) gel electrophoresis difficult.

Oxidation of sinapyl alcohol by peroxidases and the effect of cinnamic acids and coniferyl alcohol

The oxidation of each monolignol or cinnamic acid by peroxidases was determined (Table 1). The CWPO-C isoenzyme oxidized sinapyl alcohol at about 18 times the rate of CWPO-A and HRP. Similarly, this peroxidase oxidized sinapic acid better than ferulic acid. On the other hand, the rate of sinapyl alcohol oxidation by CWPO-C was not affected by addition of coniferyl alcohol. The substrate preferences of CWPO-A and HRP were similar, and both peroxidases hardly catalyzed the oxidation of syringyl substrates. To our knowledge, CWPO-C has the greatest preference for sinapyl alcohol substrate ever reported.[15,27–29] It seems likely that CWPO-C has a unique way of catalyzing the oxidation of syringyl-type aromatic structures.

The degree to which each peroxidase oxidized sinapyl alcohol was determined in the presence of cinnamic acids at half the concentration of sinapyl alcohol (Table 1). The oxidizing activity of CWPO-A and HRP increased 9-fold and 25-fold, respectively, with the addition of ferulic acid. This result is consistent with the observation reported by Takahama et al.[22,23] In contrast, the addition of cinnamic acids to the reaction mixture did not greatly affect the oxidation of sinapyl alcohol by CWPO-C. We also examined the effect of coniferyl alcohol on the oxidation of sinapyl alcohol by peroxidases (Fig. 2). Coniferyl alcohol (0.01 mM) at a concentration one-tenth that of sinapyl alcohol affected the oxidation rate of sinapyl alcohol by HRP or CWPO-A, and these oxidation rates were competitive with that by CWPO-C. When 0.05 mM coniferyl alcohol (one-half the concentration of sinapyl alcohol) was added to the mixture, the rate of sinapyl alcohol oxidation increased to 1500 μmol/min·mg protein, almost same as the coniferyl alcohol oxidation rate by HRP or CWPO-A (Table 1, Fig. 2).

On the other hand, the rate of sinapyl alcohol oxidation by CWPO-C was not affected by addition of coniferyl alcohol. As shown in Fig. 3, the HPLC assay confirmed that coniferyl alcohol consumption was minimal or negligible, although the amount of sinapyl alcohol decreased owing to the peroxidase reaction. This result strongly suggests that coniferyl alcohol acts as a radical mediator for sinapyl alcohol and peroxidase, probably in the following manner: Coniferyl alcohol is first oxidized by peroxidase to form a

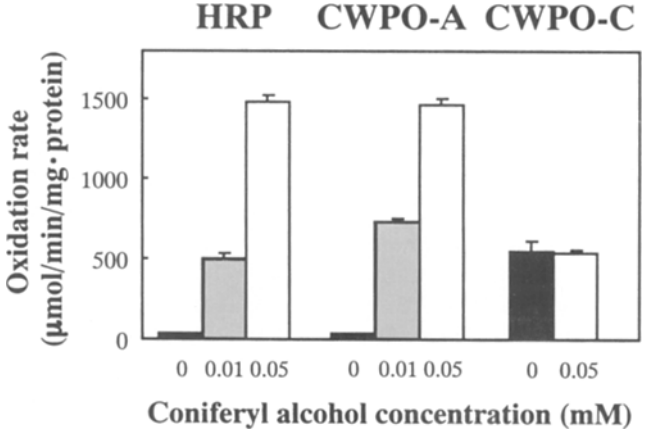

Fig. 2. Effect of coniferyl alcohol concentration on sinapyl alcohol oxidation rate by peroxidases. The oxidation rate of sinapyl alcohol (0.1 mM) was measured in the presence of 0.01 mM (*gray bars*) and 0.05 mM (*white bars*) coniferyl alcohol or the absence (0 mM, *black bars*) of coniferyl alcohol

Table 1. Oxidation of monolignols and cinnamic acids by peroxidases

Substance	Monolignols		Cinnamic acids		Sinapyl alcohol oxidation with	
	Coniferyl alcohol	Sinapyl alcohol	Ferulic acid	Sinapic acid	Ferulic acid	Sinapic acid
CWPO-C	433 ± 24	705 ± 13	186 ± 8	927 ± 38	772 ± 15	1051 ± 16
CWPO-A	1572 ± 37	38 ± 3.0	454 ± 6	11 ± 1.4	353 ± 3	36 ± 4.8
HRP	1526 ± 49	40 ± 0.6	1053 ± 10	8.9 ± 0.7	996 ± 56	38 ± 1.6

Oxidation rate was expressed as consumed substrate (μmol/min·mg protein). Data are means of triplicate analyses ± SD
CWPO-C, cationic peroxidase; CWPO-A, anionic peroxidase; HRP, horseradish peroxidase

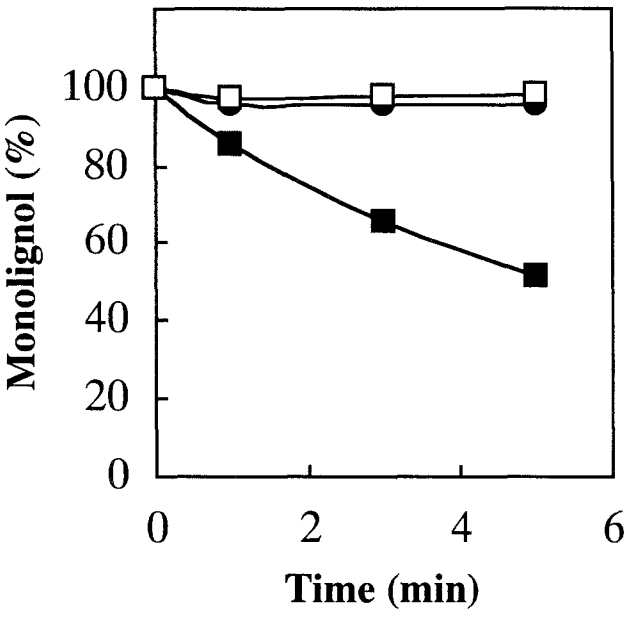

Fig. 3. Monolignol consumption during the horseradish peroxidase (HRP) reaction. The reaction mixture contained 0.15 mM of each sinapyl alcohol and coniferyl alcohol. Consumption of coniferyl alcohol (*circles*) in the presence of sinapyl alcohol. Consumption of sinapyl alcohol in the presence (*filled squares*) or absence (*open squares*) of coniferyl alcohol

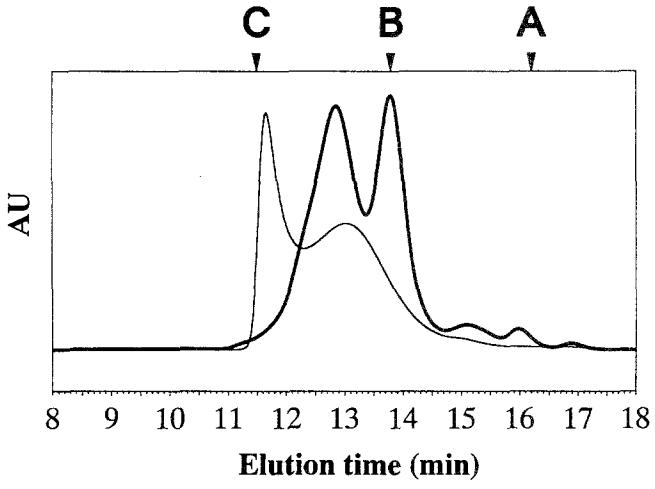

Fig. 4. Gel permeation chromatogram of synthesized S-oligo and dehydrogenative polymer (DHP) of coniferyl alcohol. S-oligo (*bold line*) and coniferyl-DHP (*regular line*) were separated on an α-2500 column with a dioxane/water mixture (4:1) containing 40 mM LiCl. The elution times of sinapyl alcohol, syringaresinol, and void volume are indicated at positions *A* (16.2 min), *B* (13.8 min), and *C* (11.5 min). *AU*, absorbance units

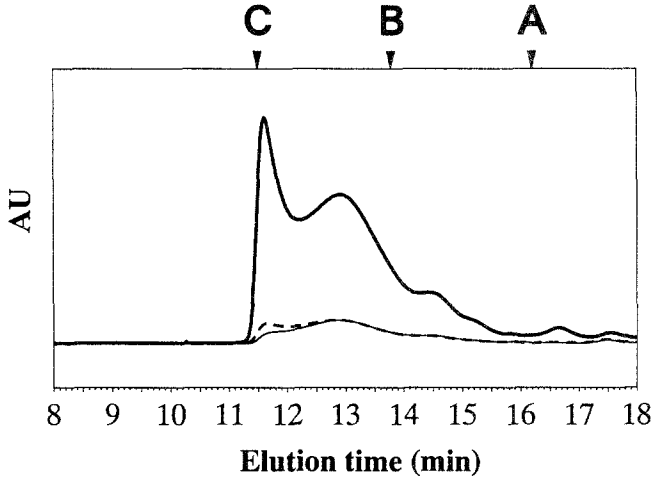

Fig. 5. Gel permeation chromatogram of the dehydrogenative polymers. S-oligo and sinapyl alcohol were reacted by CWPO-C or HRP. Water-insoluble material obtained from the CWPO-C reaction or the HRP reaction mixture was analyzed by gel permeation chromatography (GPC). *Bold line*, 0.6 μg of CWPO-C; *regular line*, 12 μg of HRP; *broken line*, 60 μg of HRP. For markers A, B, and C refer to Fig. 4

free radical, which then attracts a single electron from sinapyl alcohol to produce a sinapyl alcohol-free radical while simultaneously reforming the coniferyl alcohol molecule. Our results indicate that peroxidase isoenzymes, such as HRP and CWPO-A, which do not often use sinapyl alcohol as substrate, can catalyze single-electron oxidation of sinapyl alcohol using coniferyl alcohol as a radical mediator. In addition, any peroxidase isoenzyme might be able to catalyze sinapyl alcohol oxidation when appropriate radical mediators are present.

Polymerization of S-oligo by peroxidase

As Sarkanen stated, an "end-wise" polymer containing many β-O-4 linkages can be formed by coupling monolignols to a growing polymer one by one.[30] Essential to this hypothesis is that peroxidase catalyzes the oxidation of oligomeric or polymeric lignols to form their radicals. To determine the catalytic efficiency with which peroxidases oxidatively polymerize oligomeric lignols, we prepared S-oligo via peroxidase action, as described in Materials and methods. The S-oligo elution profile from the GPC analysis showed that it has two molecular-weight distributions (Fig. 4). The elution time (13.8 min) of the later peak was consistent with that of authentic syringaresinol, which is a β-β-coupled sinapyl alcohol dimer. For the comparison, the elution profile of the synthesized dehydrogenative polymer from coniferyl alcohol by the Zutropf method (coniferyl-DHP) is also shown in Fig. 4; the major peak was eluted at 11.5 min, which corresponded to the void volume of this column. Thus, the peak that eluted between 11.5 and 13.5 min in S-oligo was confirmed to be an oligomeric fraction.

According to the results shown in Table 1, CWPO-A and HRP oxidize monolignols similarly. Therefore, we further investigated the polymerization of S-oligo and sinapyl alcohol using CWPO-C and HRP. In these experiments, a reaction mixture containing S-oligo and sinapyl alcohol (1:1 w/w) was catalyzed by CWPO-C and HRP, and then the molecular weight distributions of the reaction products were compared using GPC (Fig. 5). In the reaction mediated by 0.6 μg of CWPO-C, the peaks corresponding to sinapyl alcohol and syringaresinol disappeared. The elution

time of the reaction product shifted from 12.8 to 11.5 min, and this elution time corresponds to the void volume of the column. The profile of the reaction product was similar to the profile of coniferyl-DHP shown in Fig. 4. Thus, GPC indicates that CWPO-C catalyzed the coupling of S-oligo and sinapyl alcohol, which produced polymers with a molecular weight greater than that of S-oligo. However, in the reaction mediated by 12 and 60 μg of HRP, which are 20-fold and 100-fold the amount of CWPO-C, respectively, the amount of formed water-insoluble material was quite small (Fig. 5). Furthermore, the area of the high-molecular-weight region of the product was much less than that of the CWPO-C reaction. When the elution profile of the whole reaction mixture was subjected to GPC analysis, a peak corresponding to a sinapyl alcohol dimer (elution time 13.8 minutes) greatly increased, but no polymers of high molecular weight were observed (data not shown). These results clearly suggest that during the HRP reaction sinapyl alcohol and S-oligo rarely coupled, but sinapyl alcohol was converted to dimeric compounds. This observation supports the suggestion that HRP-induced coupling of sinapyl alcohol yields a large amount of syringaresinol.[31] The result in Fig. 5 suggests that CWPO-C is capable of mediating the oxidation of both S-oligo and sinapyl alcohol, as well as the dehydrogenative polymerization that produces lignin polymers of higher molecular weight.

As shown in Fig. 2, HRP was able to oxidize sinapyl alcohol in the presence of coniferyl alcohol as a radical mediator. We also examined whether S-oligo and sinapyl alcohol are polymerized in the presence of coniferyl alcohol by HRP. In this experiment, we used low sinapyl alcohol/coniferyl alcohol molar ratios (1.00:0.06 and 1.00:0.12) as radical mediators because (1) one-tenth of the molar ratio was enough to ensure sinapyl alcohol oxidation (Fig. 2), and (2) the coniferyl alcohol polymer formed by HRP action would not affect monitoring changes in the S-oligo GPC profile. In the presence of coniferyl alcohol, the peak appeared between 12 and 13 min, corresponding to oligomers, compared to the mixture that lacked coniferyl alcohol (Fig. 6). The amount of oligomer increased with the coniferyl alcohol concentration, although no material of larger molecular weight than oligomers was observed in the GPC profiles. This result strongly suggests that the coniferyl alcohol radicals rarely transfer to S-oligo. Furthermore, the increase in the yield of oligomers compared to the HRP-mediated reaction suggests that the coniferyl alcohol radical can mediate the oxidation of sinapyl alcohol and small-molecular-weight oligomers such as dimers, trimers, and so on; consequently, sinapyl alcohol oligomers accumulated in the reaction mixture but high-molecular-weight polymer was not produced.

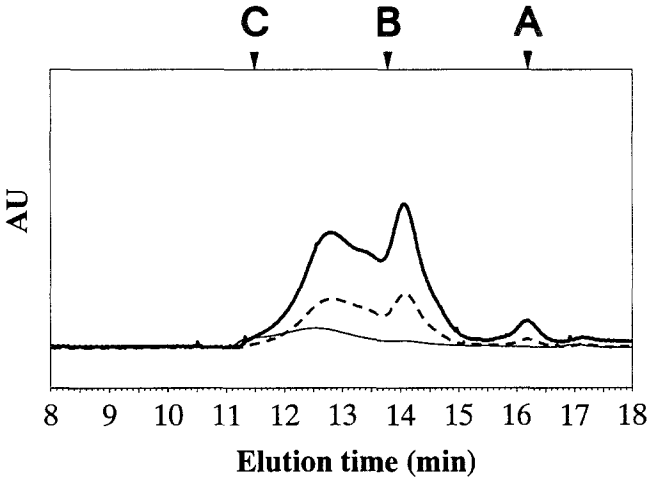

Fig. 6. Gel permeation chromatogram of the final product of S-oligo and sinapyl alcohol polymerized by HRP in the presence of coniferyl alcohol. The peroxidase reaction proceeded in the absence of coniferyl alcohol with 12 μg of HRP or in the presence of 0.04 mg or 0.08 mg of coniferyl alcohol with 0.6 μg of HRP. The sinapyl alcohol (0.8 mg)/coniferyl alcohol molar ratio was 1.00:0.12 (0.08 mg) or 1.00:0.06 (0.04 mg). *Bold line*, coniferyl alcohol (0.08 mg) and HRP (0.6 μg); *broken line*, coniferyl alcohol (0.04 mg) and HRP (0.6 μg); *regular line*, no coniferyl alcohol but HRP (12 μg). For markers A, B, and C refer to Fig. 4

Discussion

Sinapyl alcohol is more reactive than coniferyl alcohol upon chemical oxidation, and inorganic oxidants such as aqueous $FeCl_3$ are better catalysts of sinapyl alcohol than of coniferyl alcohol or *p*-coumaryl alcohol. This behavior is strongly supported by the molecular orbital calculation. However, the situation is reversed for the oxidation of monolignols catalyzed by peroxidases. The best-studied peroxidase, HRP, barely catalyzes the oxidation of sinapyl alcohol, as shown in Table 1. Similarly, most peroxidases either have low substrate preference or low activity toward sinapyl alcohol compared with coniferyl alcohol,[15,27–29] likely because of the enzyme–substrate interaction between peroxidase and monolignols. Alternatively, sinapyl alcohol may not be a good substrate for most studied peroxidases, although peroxidases usually can use a wide range of substrates. We found CWPO-C to be a highly hydrophobic peroxidase that, unlike other peroxidases, prefers sinapyl alcohol to coniferyl alcohol as a substrate. Its strong ability to oxidize sinapyl alcohol may be related to the fact that it is hydrophobic or that it has an unusual mechanism of substrate preference compared with other peroxidases.

There is a dearth of information about polymerization by peroxidase isoenzymes of monolignols to high-molecular-weight lignin.[13,20,21] Considering that monolignol polymerization occurs by an "end-wise" process, monolignol radicals should couple with prepolymers (oligomers) to produce a lignin macromolecule. Thus, the peroxidase responsible for lignification should be able to catalyze single-electron oxidation of either monomeric or oligomeric lignols. In this study, polymerization of S-oligo by peroxidases was measured by GPC analyses. HRP and CWPO-A, which did not use sinapyl alcohol preferentially as a substrate, oxidized sinapyl alcohol when coniferyl alcohol was available to act as a radical mediator. As shown in Fig. 6, however, these peroxidases barely catalyzed the polymerization of S-oligos and sinapyl alcohol in the presence of

coniferyl alcohol. These results suggest that the coniferyl alcohol radical may be hardly transferred to the S-oligo, whereas the radicals can be quickly transferred from coniferyl alcohol to sinapyl alcohol. The cationic peroxidase bound to cell walls, CWPO-C, preferred sinapyl alcohol to coniferyl alcohol as a substrate and could catalyze the in vitro polymerization of S-oligo and sinapyl alcohol. This result suggests that CWPO-C can interact with S-oligo and catalyze oxidation of the oligomers. Johjima et al. investigated the interaction between lignin peroxidase and synthetic lignin (the dehydrogenative polymer of coniferyl alcohol) in relation to lignin biodegradation.[32] They observed the formation of a lignin peroxidase and dehydrogenative polymer complex but were unable to confirm the interaction between the polymer and other peroxidases (manganese peroxidase and HRP). In our study, S-oligo was not a pure compound but a mixture of oligomeric compounds that contains molecular weight distribution and several linkage types between phenylpropanoid (C6-C3) units. Therefore, peroxidase–low-molecular-weight substrate interaction in the access channel does not explain how CWPO-C can catalyze the oxidation of S-oligo. This syringyl-specific peroxidase probably interacts with oligomeric lignols in a novel manner, as suggested by Johjima et al.[32]

Conclusions

We demonstrated that CWPO-C is highly specific to sinapyl alcohol and catalyzes in vitro polymerization of S-oligo and sinapyl alcohol to produce a lignin polymer. Thus, we characterized CWPO-C as a possible lignification-specific peroxidase isoenzyme, especially related to the deposition of syringyl-rich lignin in the secondary cell walls of woody angiosperms.

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