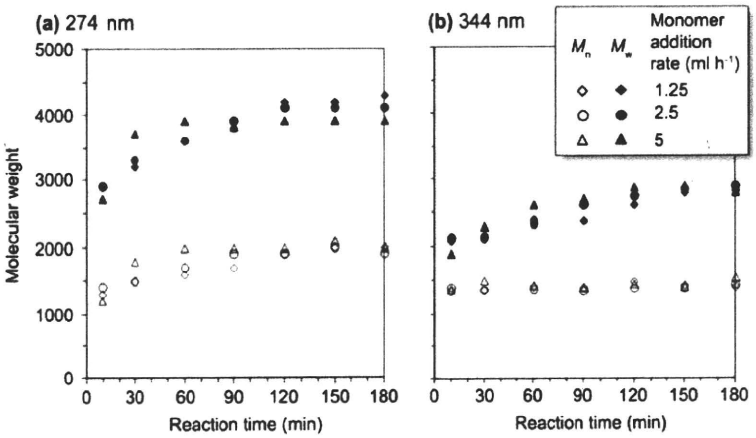

Figure 6 Product molecular weights calculated based on GPC chromatograms detected at 274 nm (a) and at 344 nm (b) during the course of HRP-catalyzed polymerization of iso-S. M_w, weight average molecular weight. M_n, number average molecular weight.

GPC-PDA monitoring of copolymerization of iso-G and iso-S

It has been recognized for many years that S-type monomers can be converted to high molecular weight DHPs in the presence of G- or H-type monomers (Freudenberg and Hübner 1952; Schweers and Faix 1973; Tobimatsu et al. 2008b). It is obvious that the presence of G- or H-type monomers greatly enhances the enzymatic oxidation rates of S-type monomers, which is generally interpreted that the former act as radical mediators from enzyme to S-type monomers (Takahama et al. 1996; Aoyama et al. 2002; Fournand et al. 2003; Sasaki et al. 2004; Hatfield et al. 2008; Tobimatsu et al. 2008b). In copolymerization experiments with iso-G and iso-S, the accumulation of S-type QMs was suppressed, and Tobimatsu et al. (2008c) suggested that G-type monomers might also serve as nucleophiles for S-type QMs in their copolymerization. In other words, the oligomeric S-type QMs rearomatize more rapidly in the presence of iso-G and thus a subsequent polymerization of the resulting phenolic intermediates becomes possible and DHPs with higher molecular weights arise.

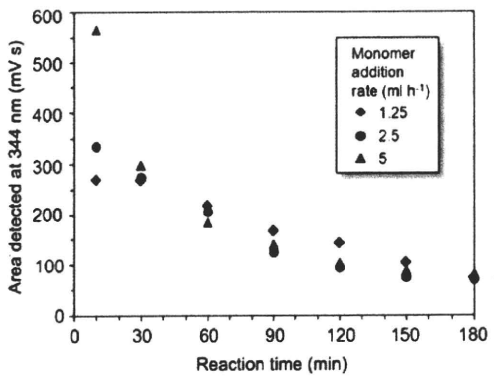

Figure 7 Plots of GPC peak area detected at 344 nm in HRP-catalyzed polymerization of iso-S.

To confirm this concept, the time dependent copolymerization of iso-G and iso-S was monitored by GPC-PDA. The concentration of iso-S was set to constant and the concentration of iso-G was systematically varied. Therefore, the potential amount of S-type QMs was the same in all monomer feed ratios.

Figure 4c displays GPC-PDA profiles of the copolymerization products at 60 min reaction time. Formations of the phenolic intermediates and S-type QMs could be detected separately by their absorptions at 274 nm and 344 nm, respectively. As readily visible, the copolymerization profiles were much affected by the feed ratios. The molecular weights calculated based on PDA detection at 274 nm and 344 nm are plotted against reaction time in Figure 8. The increasing iso-G content in the monomer mixtures contributed to the increment of molecular weights of phenolic intermediates (Figure 8a). It is obvious that the direct participation of iso-G plays an important role for the polymerization of iso-S and accelerates the formation of DHPs with high molecular masses. The molecular weights calculated based on 344 nm detection also increased systematically with increasing iso-G content (Figure 8b). In addition, it was clearly observed that the GPC peak areas detected at 344 nm were reduced with increasing iso-G content as shown in Figure 9. This indicates that accumulations of S-type QMs intermediates are suppressed in the presence of iso-G.

The data presented here fully support the concept suggested in our previous studies. It is obvious that QMs rearomatize more rapidly in the copolymerization of G-type and S-type monomers than in the homopolymerization of S-type monomers and leads to DHPs with higher molecular masses. Two mechanisms are conceivable for interpretation: (1) higher reactivity of G/S-type QMs resulting from cross-couplings between G- and S-type substrates and (2) high nucleophilicity of G-type phenolic hydroxyl groups towards S-type QMs. Our previous study showed significantly high reactivity of a G-type phenolic compound toward S-type QMs and supports the second explanation (Tobimatsu et al. 2008c).

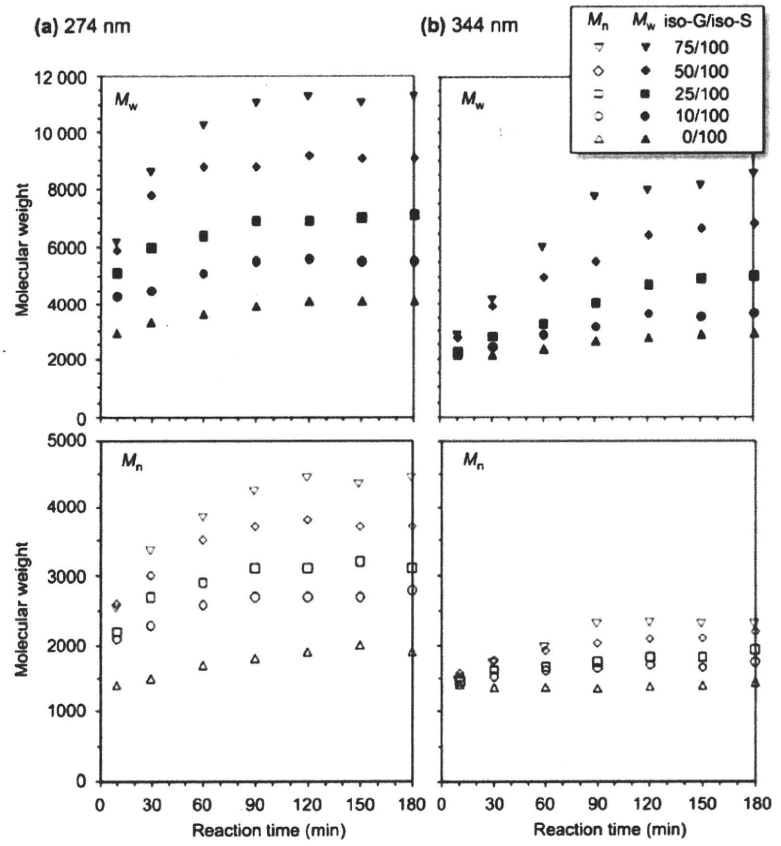

Figure 8 Product molecular weights calculated based on GPC chromatograms detected at 274 nm (a) and at 344 nm (b) during the course of HRP-catalyzed copolymerization of iso-G and iso-S. M_w, weight average molecular weight. M_n, number average molecular weight.

Obviously, the results of a copolymerization between G- and S-type monomers should be a high abundance of non-cyclic α-aryl ether bonds in DHPs. Indeed, many authors demonstrated the predominant occurrence of non-cyclic α-aryl ethers in DHPs (Saake et al. 1996; Landucci et al. 1998; Ämmälahti and Brunow 2000). Nevertheless, it is still questionable to conclude that S-type QMs intermediates are scavenged via nucleophilic additions of G-type phenolic compounds during lignin formation in plant cell (lignification *in vivo*), because native lignins contain much less α-etherified β-O-4 substructures than non-etherified ones (Ede and Brunow 1992; Saake et al. 1996; Crestini and Argyropoulos 1997).

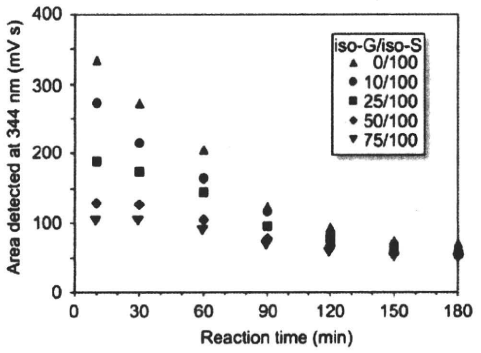

Figure 9 Plots of GPC peak area detected at 344 nm in HRP-catalyzed copolymerization of iso-G and iso-S.

Conclusions

GPC-PDA techniques have been applied to monitor the HRP-catalyzed dehydrogenative polymerization of monolignol glycosides. Working with water-soluble monolignol glycosides has the advantage of dehydrogenative polymerization in a homogeneous phase. The GPC-PDA method is suited to follow the changes of the molecular weights in the course of DHP formations. In addition, a PDA detector permits a semi-quantitative determination of the oligomeric S-type quinonemethide intermediates (S-type QMs), which are relatively stable and impede further polymerization.

Based on the results presented in this study and in previous publications (Tobimatsu et al. 2008c,d, 2009), we propose that the formation of unreactive oligomeric S-type QMs is responsible for the low yield and low molecular mass of DHP in typical dehydrogenative polymerization of S-type monomers. It seems probable that G-type monomers quench stoichiometrically the S-type QMs in copolymerization

experiments and the molecular mass of the DHP becomes higher. So far, the contributions of stable S-type QMs in DHP formations have not been the focus of much attention. Subsequent studies should focus on the reactivity of S-type QMs under various polymerization conditions. Such studies are expected to also provide new clues for understanding the factors controlling lignin polymerization *in vivo*.

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Reactivity of syringyl quinone methide intermediates in dehydrogenative polymerization. Part 2: pH effect in horseradish peroxidase-catalyzed polymerization of sinapyl alcohol

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Abstract

The solvent pH effect in the horseradish peroxidase (HRP)-catalyzed polymerization of sinapyl alcohol (S-alc) and analogously, sinapyl alcohol γ-*O*-glucoside (isosyringin, iso-S) was investigated particularly focusing on the behavior of syringyl-type quinone methide intermediates (S-type QMs) under acidic conditions. At first, the HRP-catalyzed polymerization of iso-S at pH 6.5–2.5, which produces water-soluble dehydrogenation polymer (DHP) intermediates in a homogeneous phase, was monitored by UV spectroscopy and gel permeation chromatography with photodiode array detection (GPC-PDA). Under acidic conditions at pH below 4.5, unstablilized S-type QMs from iso-S are rapidly quenched resulting in efficient productions of DHPs, although substantial loss of HRP activity and the resulting insufficient polymerization were inevitable at pH below 3.5. In addition, it was found that a small addition of guaiacyl-type comonomer (isoconiferin, iso-G) effectively promotes the polymerization of iso-S under acidic conditions, in which the comonomer serves as a radical mediator to facilitate the HRP-catalyzed oxidations of iso-S. Next, the HRP-catalyzed polymerization of S-alc at various pH values was conducted and the resulting DHPs were characterized by GPC and NMR measurements. The yields of isolated DHPs significantly increased as solvent pH decreased below 4.5. The structural analyses of the DHPs demonstrated that reaction selectivity of S-type QMs during the polymerization drastically changed at pH below 4.5: they react efficiently with water molecules as solvent leading to the formation of benzyl alcohol type β-O-4 substructures preferentially to the formation of α-O-aryl type substructures. Consequently, the data in this study demonstrated that acidic conditions at pH below 4.5 are favored in the dehydrogenative polymerization of S-alc from the viewpoint of the reactivity of S-type QMs.

Keywords: dehydrogenation polymer (DHP); horseradish peroxidase (HRP); lignin biosynthesis; nucleophilic addition; pH; quinone methide; syringyl lignin.

Introduction

The final phases of the formation of lignin in plant cell walls basically involve the enzymatic dehydrogenative polymerization of monolignols, such as coniferyl alcohol (G-alc), sinapyl alcohol (S-alc), and *p*-coumaryl alcohol (H-alc), giving rise to a complex hydrophobic network of phenylpropanoid units. For many years, the oxidative polymerization of monolignols in vitro by one-electron oxidants, such as horseradish peroxidase (HRP)/peroxide system, has been utilized as a versatile tool to gain mechanistic insights into the lignin polymerization in plant cell wall. The resulting "synthetic lignins" (dehydrogenation polymers, DHPs) has been widely used as lignin polymer models to investigate the structures and reactivity of native lignins (Brunow et al. 1998; Boerjan et al. 2003; Ralph et al. 2004, 2008; Grabber 2005).

However, it has been recognized that the in vitro dehydrogenative polymerization of syringyl (S)-type monolignol, i.e., S-alc, produces DHPs in extremely low yields, whereas polymerization of guaiacyl (G) and *p*-hydroxyphenyl (H)-type monolignols, i.e., G-alc and H-alc, easily give rise to their corresponding DHPs in high yields (Freudenberg and Hübner 1952). As well established, dehydrogenative polymerization is based on three distinct reaction types as depicted in Figure 1b: (1) enzymatic radical formations, (2) radical couplings, and (3) rearomatization of quinone methide intermediates (QMs) by nucleophilic additions of nucleophiles in the polymerization system. The subsequent repetition of the steps shown in Figure 1b would lead to polymer chains. Two essential reasons for inhibited chain polymerization of S-alc have been identified: (1) the relatively low reactivity of com-

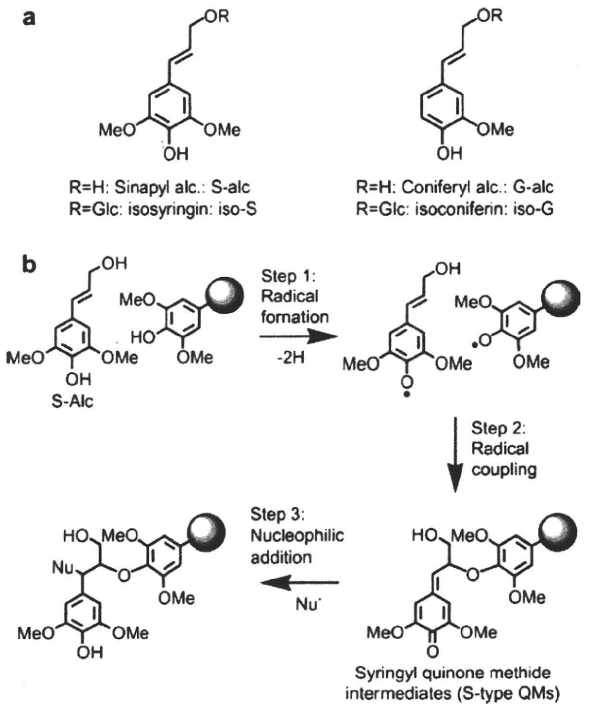

Figure 1 Chemical structures of monolignols and monolignol γ-*O*-glucosides (a) and dehydrogenative polymerization of S-alc via β-O-4 coupling (b).

mon enzymes such as HRP to S-alc in step 1 (Østergaard et al. 2000; Nielsen et al. 2001; Aoyama et al. 2002; Sasaki et al. 2004; Kobayashi et al. 2005) and (2) preferential β-β coupling reactions to β-O-4 units in step 2 (Tanahashi et al. 1976; Tanahashi and Higuchi 1990; Lu et al. 2004; Kobayashi et al. 2005).

Recently, Tobimatsu et al. (2008c, 2010a) demonstrated the high stability of oligomeric S-type QMs in step 3 by monitoring of HRP-catalyzed polymerization of monolignol γ-*O*-glucosides [i.e., isosyringin (iso-S) and isoconiferin (iso-G), Figure 1a] by UV spectroscopy and gel permeation chromatography equipped with photodiode array detection (GPC-PDA). Furthermore, it was shown that the HRP-catalyzed polymerization of S-alc produces DHPs in remarkably high yields in the presence of nucleophilic reagents such as azide ion, which served as S-type QM scavengers (Tobimatsu et al. 2008d, 2010b). These results clearly indicate that the low reactivity of S-type QMs with nucleophiles in a conventional reaction system critically impedes subsequent polymerization toward S-DHPs.

The question is, however, how S-type QMs are scavenged efficiently during the formation of S-type lignins in vivo, i.e., in plant cell walls. We previously demonstrated that S-type QMs could be quenched in the case of copolymerization of S-type and G-type monomers in vitro and suggested that G-type phenols potentially serve as good nucleophilic reactants towards S-type QMs. Non-cyclic α-aryl ether structures are present only in low amounts in native lignins (Ede and Brunow 1992; Saake et al. 1996; Crestini and Argyropoulos 1997) and therefore it is not likely that the nucleophilic additions of G-type substrates to S-type QMs are operative during lignin formation in vivo. Based on the fact that the most abundant β-O-4 structures in native lignins comprise free hydroxyl groups on their α-positions, it can be deduced that QMs are exclusively scavenged by water molecules during lignin formation.

Dehydrogenative polymerization of monolignols in vitro has been carried out generally in neutral buffers in view of the catalytic ability of the common enzymes such as HRP. By contrast, several studies with synthetic QM compounds have showed that they react quickly in acidic media possibly owing to protonations of QM, which leads to highly electrophilic benzylcations (Hemmingson and Leary 1975; Leary and Thomas 1975; Sipilä and Brunow 1991, 1992; Brunow et al. 1993; Bolton et al. 1997; Richard et al. 2000). Therefore, it is conceivable that in HRP-catalyzed polymerization of S-alc in acidic buffers, the rearomatizations of S-type QMs would be promoted and then subsequent chain polymerization steps would result in DHPs with higher mol masses and yields. So far, several authors investigated the in vitro oxidative couplings of S-alc and analogs under acidic conditions (Yoshida et al. 1994; Fournand et al. 2003), in which, however, neither the reactivity of S-type QMs nor the efficiency of DHP productions from S-alc have been the focus of much attention.

In this report, the effect of pH in the HRP-catalyzed dehydrogenative polymerization of S-alc and analogously iso-S will be described and the focus will be particularly on the behavior of S-type QMs under acidic conditions. At first, the polymerization of iso-S at pH values from 6.5 to 2.5 will be monitored by UV spectroscopy and GPC-PDA measurements, then the corresponding behavior of S-alc at various pH values will be described and the resulting DHPs will be characterized by GPC and NMR spectroscopy.

Materials and methods

Monolignol γ-*O*-glucosides, iso-S and iso-G, were synthesized according to Takano et al. (2006) and the monolignols, S-alc and G-alc, according to Quideau and Ralph (1992). HRP (100 U mg^{-1}) was purchased from Wako Pure Chemicals (Osaka, Japan) and used without further purification. 2-Chrolo-1,3,2-dioxaphospholane and 2-chrolo-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane were purchased from Sigma-Aldrich Chemical Co. (Tokyo, Japan) and the other chemicals from Nacalai Tesque Inc. (Kyoto, Japan) or Wako Pure Chemicals were used as received. The pH values of 6.5–5.5 and 4.5–2.5 were adjusted with sodium phosphate and sodium phosphate/citrate buffer solutions, respectively.

UV spectroscopic monitoring of HRP-catalyzed polymerization of iso-S at various pH values

The buffer solution (3 ml) consisting of 60 μM H_2O_2 and 100 μM iso-S, or 90 μM iso-S and 10 μM iso-G, and the other buffer solution (3 ml) were placed in a sample cell and a reference cell, respectively. The cells were set in a JASCO V-560 spectrophotom-

eter kept at 25°C and a spectrum was scanned (reaction time: 0). The polymerization was initiated by adding 25 μl of a concentrated HRP aqueous solution (final concentration: 3–15 mg l^{-1}) to both the sample and reference cells, and UV spectra were recorded every 2 min after initiating the polymerization (scan rate: 2000 nm min^{-1}; scan region: 250–400 nm; data interval: 1 nm; response mode: quick).

GPC-PDA monitoring of HRP-catalyzed polymerization of iso-S at various pH values

An aqueous solution (2.5 ml) containing iso-S (18 μmol), iso-G (2 μmol) and HRP (2 mg) and another buffer solution (2.5 ml) containing H_2O_2 (24 μmol) were simultaneously added to a 0.5-ml buffer solution at 25°C, with peristaltic pumps at 2.5 ml h^{-1} (monomer addition time: 60 min). After initiating the addition, 100 μl of the reaction mixtures was periodically sampled, mixed with 900 μl of 0.1 M LiCl in *N,N*-dimethylformamide (DMF) to terminate the reaction, cooled at 0°C and subjected to the GPC-PDA analyses within 15 min after withdrawing from the reaction system. The GPC-PDA analyses were performed on a Shimadzu LC-20A LC system (Shimadzu, Japan) equipped with a SPD-M20A photodiode array detector. Elution conditions: column TSK gel α-M (Tosoh, Japan); eluent 0.1 M LiCl in DMF; flow rate 0.5 ml min^{-1}; column oven 40°C; injection volume 20 μl. Conditions for PDA detection: scan region 260–400 nm; bandwidth 4 nm; response 1280 ms. The data acquisition and computation software: LCsolution version 1.22 SP1 (Shimadzu).

HRP-catalyzed polymerization of S-alc at various pH values

Two solutions were prepared for polymerization. Solution A: 120 ml of distilled water containing S-alc (0.5 mmol) and 48 mg of HRP. Solution B: 120 ml of a buffer containing 0.019% H_2O_2 (0.6 mmol). Solutions A and B were added dropwise to 30 ml of buffer over a period of 24 h at 25°C and kept another 24 h under stirring. The precipitate of the resulting polymer was collected by centrifugation (12 000 rpm, 10 min), washed three times with distilled water and lyophilized to obtain an S-DHP.

Copolymerization of S-alc with G-alc to yield their copolymer [S/G-(9:1)-DHP] was performed in the same manner as that for S-DHP except solution A, which was replaced by 120 ml of distilled water containing S-alc (0.45 mmol), G-alc (0.05 mmol) and 48 mg of HRP.

Structural characterization of DHPs

S-DHPs and S/G-(9:1)-DHPs were subjected to GPC and NMR spectroscopy (^{1}H- and ^{31}P-NMR). Prior to GPC and ^{1}H-NMR analyses, DHPs were acetylated according to standard protocols (Cathala et al. 2003). GPC system: Shimadzu LC-10 equipped with a UV-Vis detector (SPD-10Avp, monitoring at 280 nm). Columns: K-802, K-802.5, and K-805 (Shodex); eluent $CHCl_3$; flow rate 1.0 ml min^{-1}; column temperature 40°C. The system was calibrated with polystyrene standards (Shodex). ^{1}H-NMR: Varian INOVA300 FT-NMR spectrometer (300 MHz) in chloroform-*d* with tetramethylsilane as the internal standard (0.0 ppm). Quantitative ^{31}P-NMR analyses of S/G-(9:1)-DHPs were conducted according to Argyropoulos (1994), Granata and Argyropoulos (1995), and Akim et al. (2001). Phosphitylations of the DHPs were carried out in anhydrous pyridine containing chrominium (III) acetylacetonate as a relaxation reagent by 2-chrolo-1,3,2-dioxaphospholane and 2-chrolo-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane with dimethyl-L-tartate and cyclohexanol as an internal standard, respectively. Quantitative ^{31}P-NMR spectroscopy: Varian INOVA300 FT-NMR spectrometer (121.4 MHz), inverse gated decoupling sequence. Relaxation delay: 5 s. Two sets of 512 transients were acquired. Spectral integrations for the calculation of each functional group were performed based on the spectral data in the literature (Granata and Argyropoulos 1995; Akim et al. 2001).

Results and discussion

Monitoring of HRP-catalyzed polymerization of iso-S at various pH values

UV spectroscopic monitoring The HRP-catalyzed polymerization of iso-S in buffer solutions at pH values from 6.5 to 2.5 was carried out in a quartz cell situated in a UV-Vis spectrometer. The UV spectra of the homogeneous polymerization mixtures were directly recorded every 2 min after initiating polymerization (Figure 2). In polymerization at pH 6.5 (Figure 2a), the progress of polymerization could be traced by a decrease of the characteristic absorption at 274 nm (A_{274}). Simultaneously, a strong absorption at 325 nm (A_{325}) appeared, indicating the formation of stable S-type QMs as evidenced in our previous study (Tobimatsu et al. 2008c). By contrast, in polymerization at pH 2.5 (Figure 2b) the increase in A_{325} was not observed, whereas A_{274} smoothly decreased as the reaction time elapsed. The changes of A_{274} and A_{325} are plotted in Figure 3.

At a low concentration of HRP (3 mg l^{-1}), the decreasing rate of A_{274} dropped as the pH value descended below 3.5 (Figure 3a, left). It was reported that unfolding of the tertiary structure of HRP is induced at pH below 4 at room temper-

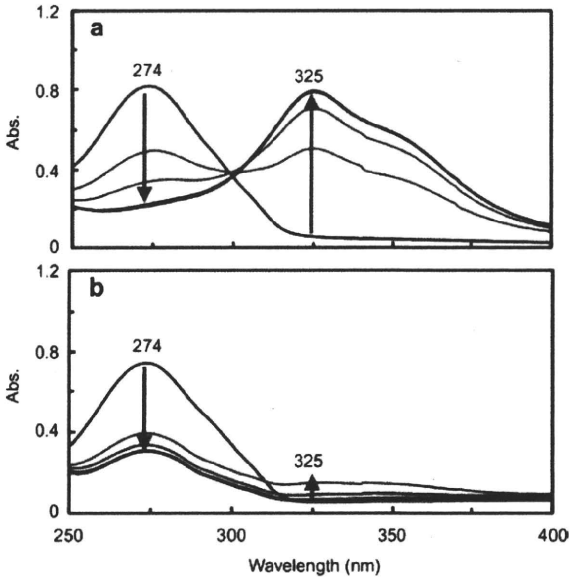

Figure 2 UV spectra during HRP-catalyzed polymerizations of iso-S at pH 6.5 (a) and at pH 2.5 (b) ([HRP] = 15 mg l^{-1}, reaction time: 0–10 min, spectra were collected every 2 min).

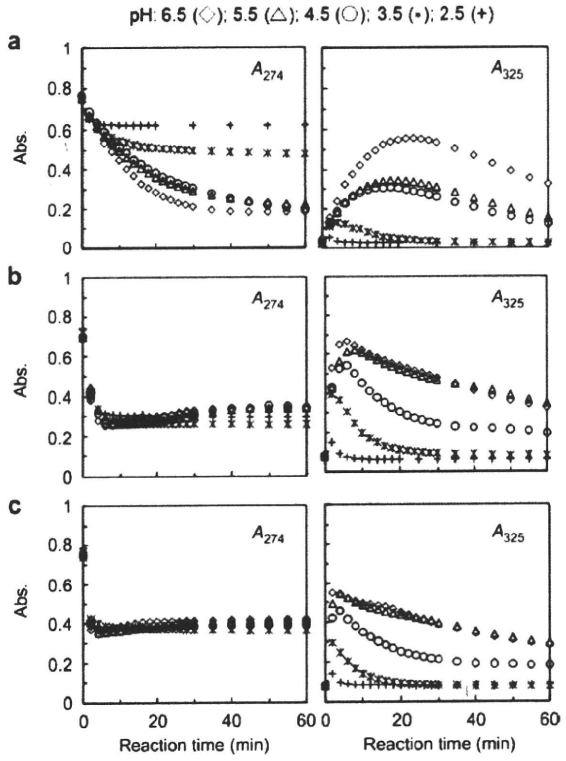

Figure 3 Changes of the absorbance at 274 nm (A_{274}) and at 325 nm (A_{325}) during HRP-catalyzed polymerizations of monolignol γ-*O*-glucosides at various pH values. Reaction conditions: (a) homopolymerization of iso-S, [HRP] = 3 mg l^{-1}; (b) homopolymerization of iso-S, [HRP] = 15 mg l^{-1}; (c) copolymerization of iso-S and iso-G (iso-S/iso-G = 9:1, mol/mol), [HRP] = 3 mg l^{-1}.

ature (Chattopadhyay and Mazumdar 2000). Expectedly, the catalytic activity of HRP to oxidize iso-S was much lower in acidic media than in neutral media. When iso-S was polymerized in a high concentration of HRP (15 mg l^{-1}), A_{274} promptly decreased in all pH ranges, indicating a smooth oxidation of the substrate even in the acidic buffers (Figure 3b, left). By contrast, the increase of A_{325} owing to S-type QMs diminished when pH values were below 4.5 (Figure 3b, right). At pH 2.5, A_{274} (iso-S) smoothly decreased, but almost no increase in A_{325} (S-type QMs) was observed.

Tobimatsu et al. (2008b) reported that iso-G as radical mediator significantly promotes the HRP-catalyzed oxidation rate of iso-S under neutral conditions. In the present study, the copolymerization of iso-S with a small amount of iso-G (iso-S/iso-G = 9:1, mol/mol in feed) was performed under acidic conditions. As expected, iso-S was effectively oxidized (A_{274} was decreasing at all pH levels) even with a low concentration of HRP (3 mg l^{-1}, Figure 4c, left). Clearly, A_{325} (S-type QMs) exhibits almost the same pH dependence as observed in the homopolymerization of iso-S (Figure 3c, right). Accordingly, iso-S can be polymerized without significant accumulation of S-type QMs in a pH range below 4.5. The presence of a small amount of G-type comonomer is enough to promote the polymerization of S-type monomers effectively under acidic conditions.

GPC-PDA monitoring The HRP promoted development of the mol mass of a mixture (iso-S/iso-G = 9:1, mol/mol in feed) was monitored by GPC-PDA. This technique also enables the simultaneous determination of oligomeric S-type QMs and polyphenolic DHP intermediates (Tobimatsu et al. 2010a). The polymerization was designed as the well-known end-wise polymerization (Sarkanen 1971; Cathala et al. 1998). The aqueous buffer solutions of the monomers, HRP and H_2O_2 were mixed at a constant rate in a period of 1 h. At a given reaction time, an aliquot of the homogeneous reaction mixtures was sampled and submitted to GPC-PDA.

Figure 4a displays the three-dimensional PDA plots of the polymerization mixtures carried out at various pH values (reaction time: 60 min). The formation of oligomeric S-type QMs in the polymerization at pH 6.5 and 4.5 was successfully detected by the characteristic absorption at 344 nm (peak top molecular weights ca. 1700), whereas the formation of polyphenolic DHP intermediates is detectable by the absorption at 274 nm. As the plots of the peak area detected at 344 nm in Figure 4b show, the amount of S-type QMs decreased faster at pH levels below 4.5. At pH 2.5, the absorptions from the S-type QMs were negligibly small. This finding is consistent with the data from UV monitoring described above. In Figure 4c, the molecular weights (MWs) of polyphenolic intermediates, calculated based on PDA detection at 274 nm, are plotted against reaction time. The MWs of polyphenolic DHP intermediates increased faster at pH 4.5 than at pH 6.5 and 5.5. Thus at pH 4.5, the efficient quenching of the oligomeric S-type QMs facilitates the subsequent production of DHPs. The promotion of the polymerization, however, was not observed in the case of iso-S at pH 3.5 and 2.5, although the reaction of S-type QMs is much faster under these acidic conditions. A possible explanation is that catalytic activity of HRP is diminished under acidic conditions to such an extent that further oxidation was impeded even if rearomatization of oligomeric S-type QMs takes place efficiently. Nevertheless, we interpreted the data that the production of DHP from S-type monomers can be facilitated at pH below 4.5 owing to the fast rearomatizations of oligomeric S-type QMs if the catalytic ability of the enzyme was maintained.

HRP-catalyzed polymerization of S-alc at various pH values

HRP-catalyzed DHP formation of S-alc at various pH values was carried out and the structures of the resulting DHPs were characterized. Analogous to the experiments with iso-S, polymerization, an unusually high HRP concentration (total 48 mg per 0.5 mmol monomer), was also applied in the presence of small amounts of G-alc as comonomer to facilitate the polymerization of S-alc under acidic conditions. Table 1 summarizes the results.

The yields of S-DHPs from homopolymerization of S-alc are significantly pH dependent. The yields under neutral conditions (pH 6.5, 5.5) are low (< 10%), but at pH 4.5 and 3.5 the yields are elevated to ca. 15% and 30%, respectively. As expected, the fast rearomatization of oligomeric S-type QM in acidic buffers promote subsequent productions of S-DHPs,

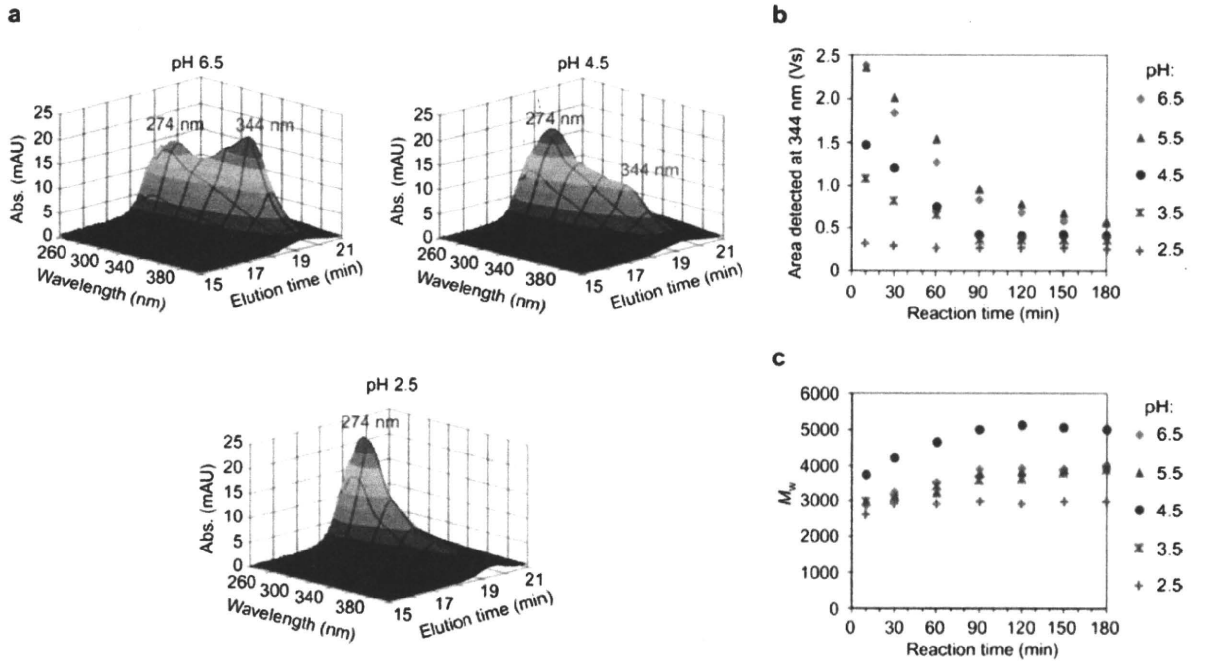

Figure 4 GPC-PDA profiles of HRP-catalyzed copolymerization of iso-S with iso-G (iso-S/iso-G = 9:1, mol/mol) at various pH values. (a) Three-dimensional PDA plots of reaction mixtures at 60 min of reaction time. (b) Plots of peak area detected at 344 nm. (c) Plots of weight average molecular weights (M_w) detected at 274 nm.

Table 1 Results from HRP-catalyzed polymerization of S-alc at various pH values.

pH of polymerization medium	Homopolymerization (S-DHPs)					Copolymerization with G-alc [S/G-(9:1)-DHPs]				
	Yield (%)	M_n[a]×10^{-3}	M_w[a]×10^{-3}	M_w/M_n[a]	DP_n[b]	Yield (%)	M_n[a]×10^{-3}	M_w[a]×10^{-3}	M_w/M_n[a]	DP_n[b]
6.5	7.7	1.6	3.0	1.9	5.3	21.4	1.7	4.0	2.4	5.9
5.5	4.3	1.8	3.0	1.7	6.2	19.0	2.0	4.5	2.3	6.9
4.5	15.2	1.5	3.3	2.2	5.0	27.7	2.2	5.9	2.8	7.7
3.5	30.5	1.8	4.0	2.3	6.2	62.1	2.2	4.6	2.0	7.7
3.0	15.0	1.8	3.3	1.8	6.2	42.8	2.0	5.0	2.5	6.9
2.5	4.9	1.7	4.0	2.4	5.9	20.5	1.8	4.6	2.5	6.2

[a]Determined by GPC after acetylation, polystyrene standards.
[b]Calculated based on the molecular weight of S-alc diacetate.

At pH 3.0 and 2.5, the yields of S-DHPs dropped again, probably as a result of the inevitable acid-induced inactivation of HRP.

The copolymerization of S-alc with a small amount of G-alc (S-alc/G-alc = 9:1, mol/mol in feed) yielded S/G-(9:1)-DHPs in much higher yields than those observed in homopolymerization of S-alc. It is obvious that S/G-DHP formation is pH dependent in a similar manner as was the case for S-DHPs. The maximum yield of S/G-DHPs is 62% at pH 3.5.

The average molecular weights (M_n and M_w) and their dispersity data (M_w/M_n) are listed in Table 1.These molecular masses of the DHPs are almost in the same range as those reported for conventional DHPs (Faix et al. 1981; Cathala et al. 1998).

In summary, HRP-catalyzed polymerization of S-alc at pH around 3.5 gives DHPs in high yields. This is probably a consequence of the fast rearomatization of oligomeric S-type QMs both formed from S-alc and iso-S. The addition of small amounts of G-type comonomer into the polymerization system effectively promotes polymerization of S-type monomers under acidic conditions. There is a small difference in this regard between S-alc and iso-S: the former yielded DHPs most efficiently at pH 3.5 and the latter at pH 4.5.

Structural characterization of S-DHPs and S/G-(9:1)-DHPs

Figure 5 shows the expanded ^{1}H-NMR spectra of acetylated DHPs, which confirm great similarity in the chemical struc-

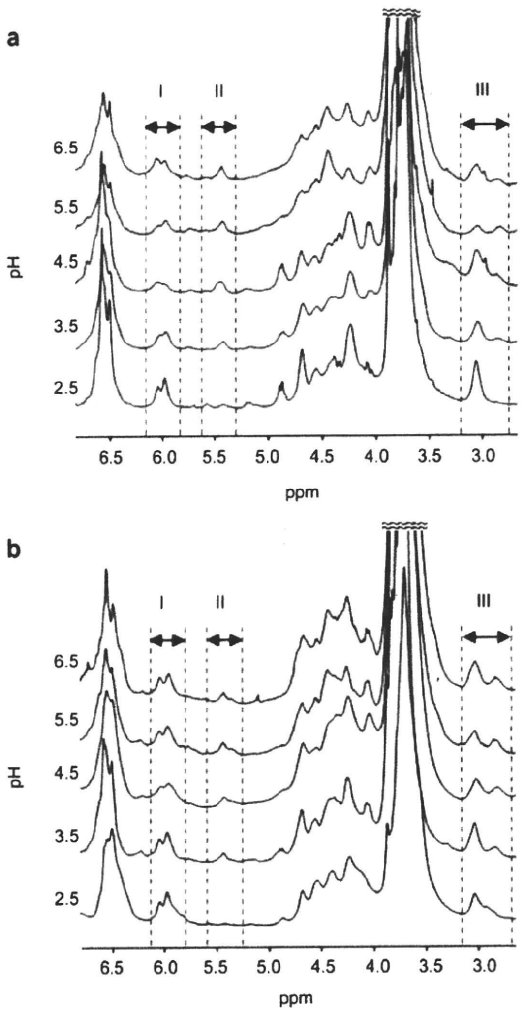

Figure 5 ^{1}H-NMR spectra of acetylated S-DHPs (a) and S/G(9:1)-DHPs (b). For abbreviations see Figure 8.

tures of S-DHPs and S/G-(9:1)-DHPs. The main substructures are indicated by the signals from α-protons in β-O-4/α-OH (**I**) (5.7–6.1 ppm), α-protons in β-O-4/α-OAr (**II**) (5.3–5.6 ppm), and β- and β′-protons in β-β resinol (**III**) (2.8–3.2 ppm), which are well suited for calculations of relative abundances of these substructures (Yamasaki et al. 1976; Brunow and Lundquist 1980; Brunow et al. 1989). In the case of S/G-(9:1)-DHPs, the portions of structure **II** are possibly overestimated owing to the signal overlapping from β-5 structures which are derived from the couplings of G-alc. No significant signal contributions from olefinic end structures are observed in the spectra. Obviously, dimerization reactions at the initial stage of polymerization take place exclusively via β-β couplings, resulting in a complete saturation of the double bond in the propanoid side chains. The preferential β-β dimerization in oxidative couplings of S-alc is well documented in the literature (Tanahashi et al. 1976; Tanahashi and Higuchi 1990; Lu et al. 2004; Kobayashi et al. 2005).

Table 2 summarizes the relative abundances of the three main structures (**I**, **II**, and **III**). It is evident that pH does not significantly affect the total amount of β-O-4 structures (**I**+**II**) and β-β structures (**III**) in S-DHPs. Obviously, the selectivity in oxidative couplings of S-alc which leads to β-O-4 and β-β structures is not affected much by the acidity of the solvent, which has already been pointed out by Yoshida et al. (1994). By contrast, the ratio of the two types of β-O-4 structures (**I**/**II**) dramatically increases at pH below 4.5 because essentially more β-O-4 structures with free benzylic OH (**I**) arise. It is probable that β-O-4 structures in S-DHP prepared at pH 2.5 mostly comprise structure (**I**). The data for S/G-DHPs are similar to that of S-DHPs.

To scrutinize the above results, the amount of hydroxyl groups in S/G-DHPs were quantified by ^{31}P-NMR spectroscopy (Argyropoulos 1994) (sufficient amounts of S-DHPs were not available). According to the methods described by Granata and Argyropoulos (1995) and Akim et al. (2001), the amount of secondary hydroxyl groups was calculated based on the spectra of derivatized DHPs with 2-chrolo-1,3,2-dioxaphospholane (Figure 6a), and the amounts of total aliphatic hydroxyl groups and phenolic hydroxyl groups were calculated from the spectra of DHPs derivatized with 2-chrolo-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (Figure 6b). The amount of OH_{prim} was quantified by subtracting the amount of OH_{sec} from the amount of OH_{tot}. Figure 7 illustrates the results.

Table 2 Relative abundances of linkage patterns in DHPs estimated by ^{1}H-NMR.

Linkage pattern		Integration range (ppm)	S-DHPs pH 6.5	pH 5.5	pH 4.5	pH 3.5	pH 2.5	S/G-(9:1)-DHPs pH 6.5	pH 5.5	pH 4.5	pH 3.5	pH 2.5
β-O-4	Total (**I**+**II**)	5.35–5.60, 5.90–6.15	66	69	63	66	67	66	70	72	70	60
	α-OH (**I**)	5.90–6.15	42	39	37	48	57	41	41	46	55	55
	α-O-Ar (**II**)	5.35–5.60	24	30	26	18	8	25	29	26	15	5
	(**I**/**II**)	–	(1.7)	(1.3)	(1.4)	(2.7)	(8.4)	(1.6)	(1.4)	(1.8)	(3.7)	(11)
β-β (**III**)		2.80–3.20	34	31	37	34	42	34	30	28	30	40

[a]β-O-4 (**I**+**II**)+β-β (**III**)=100.

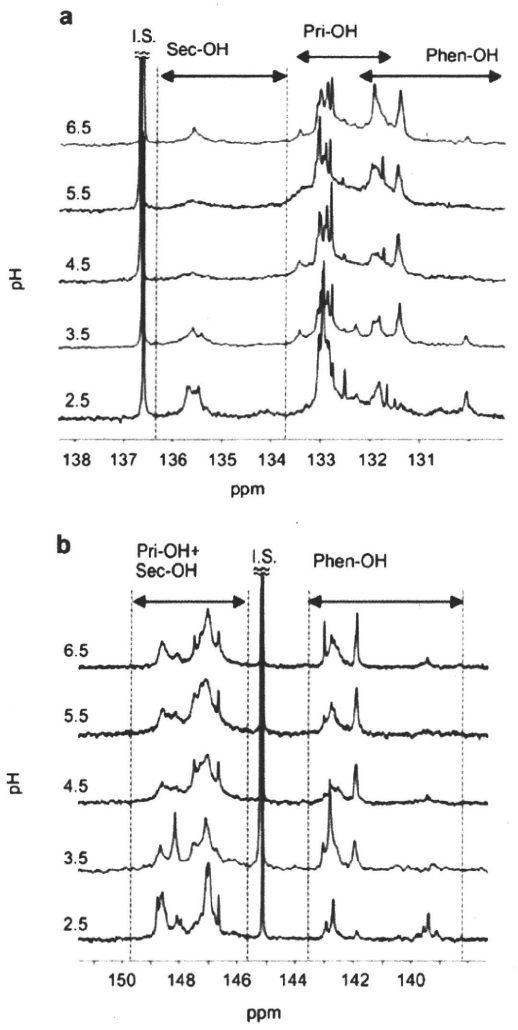

Figure 6 Quantitative ^{31}P-NMR spectra of phosphitylated S/G(9:1)-DHPs derivatized with 2-chrolo-1,3,2-dioxaphospholane (a) and 2-chrolo-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (b).

Although the amounts of OH_{prim} and OH_{phen} were not influenced much by pH, the amount of OH_{sec} markedly increased below pH 4.5. Basically, the OH_{sec} corresponds to the β-O-4 linkages with OH group on the α-C (structure **I**). In agreement with ^{1}H-NMR data, ^{31}P-NMR spectroscopy shows the increment of β-O-4 structure with OH group on the α-C at pH below 4.5.

Conclusions

There are two QM types formed during polymerization of S-alc: (1) via β-β coupling [S-type QM (β-β)] or (2) via β-O-4 coupling [S-type QM (β-O-4)] (Figure 8). The former is readily scavenged by intramolecular nucleophilic additions of γ-hydroxyl groups leading to the formation of β-β resinol structures (**III**). By contrast, S-type QM (β-O-4) are scavenged mainly by intermolecular nucleophilic additions by solvent water or phenols in oligomeric DHP intermediates. The nucleophilic additions of the former produce β-O-4/α-OH structures (**I**), whereas the additions of the latter produce β-O-4/α-OAr structures (**II**). The data from the NMR studies on the DHPs demonstrate that the reaction selectivity of S-type QM (β-O-4) dramatically changes around pH 4.5 of the solvent acidity. Under neutral or weakly acidic conditions (pH > 4.5), the frequencies of β-O-4 (**I**) are relatively low because of the prevalent nucleophilic addition of phenols, which leads to α-OAr linkages in the neighborhood β-O-4 linkages, although the presence of OH groups in the solvent is overwhelming. With lowering the pH below 4.5, the addition of OH groups is increasingly preferred.

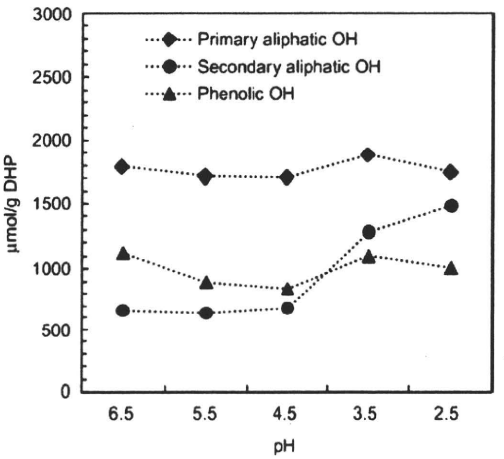

Figure 7 Quantification of primary aliphatic (◆), secondary aliphatic (▲) and phenolic (●) hydroxyl groups in S/G(9:1)-DHPs by quantitative ^{31}P-NMR.

Accordingly, S-DHPs and S/G-(9:1)-DHPs prepared under acidic conditions resembles more native lignins than those prepared under neutral conditions as pointed out by Sipilä and Brunow (1991, 1992) based on experiments with synthetic G-type QM in aqueous media. Based on biomimetic polymerization experiments with G-alc in cell wall matrix, Grabber et al. (2003) suggested that cell wall crosslinking increased below pH 4. The present study confirms that the results found for G-DHP formation are similar for S- and S/G-DHPs.

Consequently, present data demonstrated that acidic conditions at pH below 4.5 are effective for efficient polymerization of S-alc giving rise to DHPs resembling native lignins. Although the reported pH values of the plant tissues are usually between 5 and 6.5 (Grignon and Sentenac 1991), the actual pH of lignifying tissues is unknown. The low pH environment might be a possible factor controlling the formations of syringyl lignins in vivo.

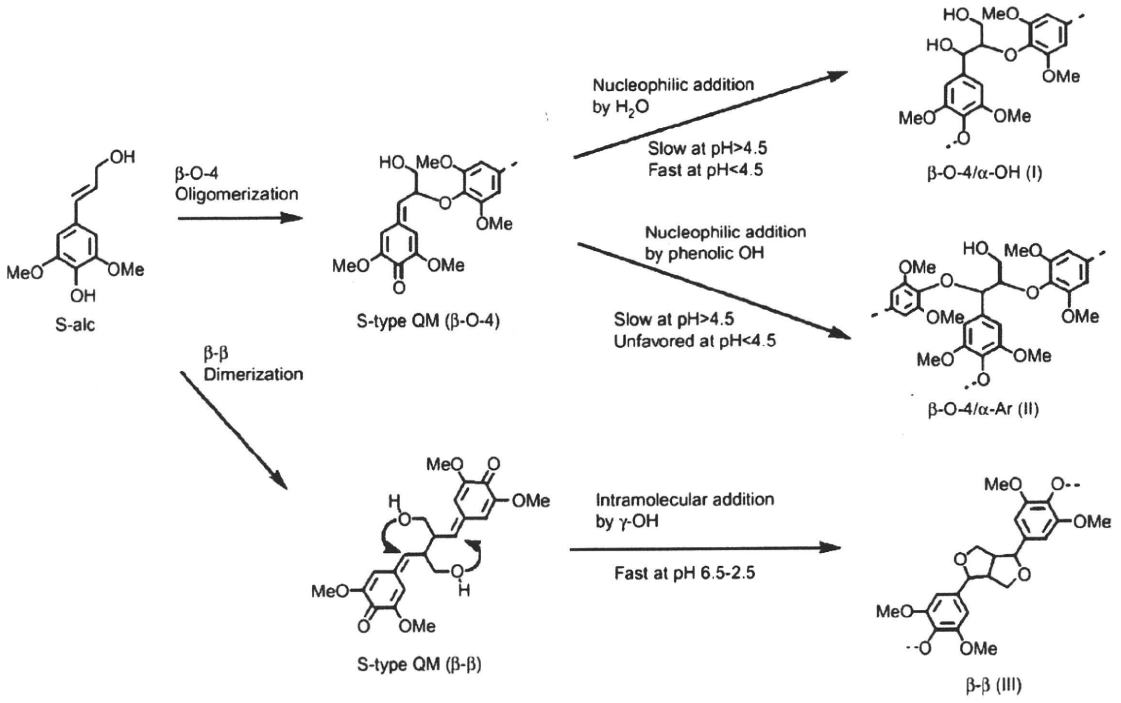

Figure 8 Reactions of S-type QMs to form the main structures in dehydrogenative polymerization of S-alc.

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ORIGINAL ARTICLE

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Reactivity of syringyl quinone methide intermediates in dehydrogenative polymerization I: high-yield production of synthetic lignins (DHPs) in horseradish peroxidase-catalyzed polymerization of sinapyl alcohol in the presence of nucleophilic reagents

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Abstract It is known that the conventional dehydrogenative polymerization of sinapyl alcohol (S-alc) gave syringyl synthetic lignins (S-DHPs), but in extremely low yields. In this article, to examine the contribution of syringyl quinone methide intermediates (S-QM) on S-DHP production, horseradish peroxidase (HRP)-catalyzed dehydrogenative polymerization of S-alc was carried out in the presence of nucleophilic reagents that promote the rearomatization of S-QM. First, the HRP-catalyzed polymerization of sinapyl alcohol γ-*O*-β-D-glucopyranoside (isosyringin, iso-S), which allows us to monitor the polymerization process in a homogeneous aqueous phase, was utilized for screening of a nucleophile used as an S-QM scavenger. Monitoring of iso-S polymerization in the presence of various nucleophilic reagents by UV spectroscopy and gel permeation chromatography with photodiode array detection (GPC-PDA) revealed a high ability of azide ion to convert oligomeric S-QM efficiently to S-DHP. Accordingly, azide ion was utilized as an S-QM scavenger in HRP-catalyzed polymerization of S-alc, which resulted in high-yield production of S-DHPs (~83%), as expected. The ^{1}H-, ^{13}C-, and 2D-HSQC NMR investigations on the resulting S-DHPs clearly demonstrated that azide ion efficiently performed nucleophilic additions to the C-α of S-QM during the polymerization. These results provide experimental proof that the low reactivity of S-QM with nucleophiles (such as water, phenolic, and aliphatic hydroxyl groups) in the conventional polymerization system critically impedes the production of S-DHPs from S-alc.

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Key words Dehydrogenation polymer (DHP) · Horseradish peroxidase (HRP) · Nucleophilic addition · Quinone methide · Syringyl lignin

Introduction

The last stage of lignin formation in the plant cell wall can be mimicked in vitro by the enzymatic dehydrogenative polymerization of monolignols [*p*-coumaryl alcohol (H-alc); coniferyl alcohol (G-alc); sinapyl alcohol (S-alc)], leading to the lignin polymer models (dehydrogenation polymers, DHPs).[1–3] As reviewed by several authors,[4–8] much of what is now known about lignin polymerizations is based on the studies of this system. However, a satisfactory synthesis of DHPs structurally resembling native lignins has not been achieved yet, implying that the polymerization process is not fully understood. One of the open questions with this regard is the peculiar polymerization behavior of S-alc, being completely different from those of H-alc and G-alc. Many researchers have reported that enzymatic dehydrogenative polymerization of S-alc afforded syringyl (S)-DHPs, but with low molecular masses in low yields, whereas H-alc and G-alc readily gave *p*-hydroxyphenyl (H)- and guaiacyl (G)-DHPs, respectively, with high molecular masses in high yields.[9–15]

As well established, the dehydrogenative polymerization of S-alc basically consisting of three reaction steps, as depicted in Fig. 1: step 1, enzymatic radical formations; step 2, radical couplings; step 3, rearomatization of syringyl quinone methide intermediates (S-QM) by nucleophilic additions of nucleophiles in the polymerization system. Several problems in each reaction step have been discussed in connection with the low polymerizability of S-alc: the low reactivity of common oxidants such as horseradish peroxidase (HRP)/hydrogen peroxide to S-type phenolic compounds for step 1[16–20] and preferential β–β coupling reactions to β-*O*-4 for step 2.[20–22] So far, little attention has been paid to step 3 in connection with the low polymerizability of S-alc in vitro.

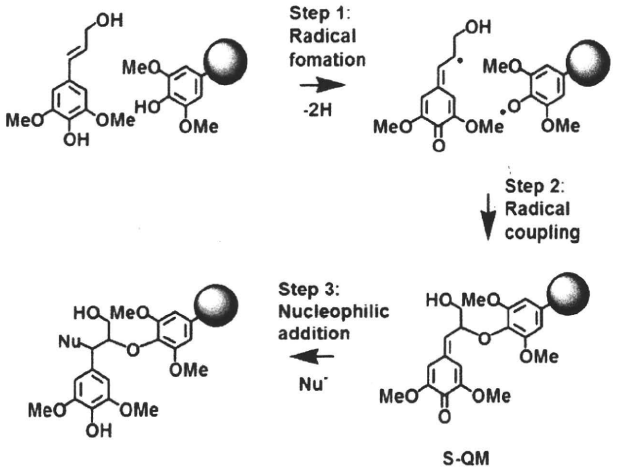

Fig. 1. Dehydrogenative polymerization of sinapyl alcohol (S-alc) via *β-O*-4 couplings

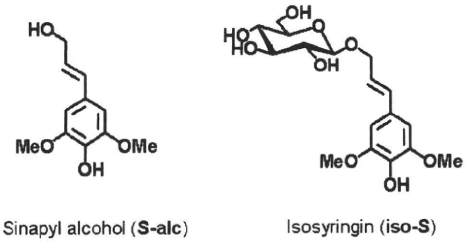

Fig. 2. Chemical structures of sinapyl alcohol (*S-alc*) and isosyringin (*iso-S*) (sinapyl alcohol γ-*O*-β-D-glucopyranoside)

Recently, we have investigated the HRP-catalyzed polymerization of sinapyl alcohol γ-*O*-β-D-glucopyranoside [isosyringin (iso-S); Fig. 2] as a model reaction system to study the polymerization behavior of S-alc.[15,23–27] Owing to the presence of a highly hydrophilic sugar unit attached to S-alc, the polymerization of iso-S gives water-soluble products in a homogeneous aqueous phase, whereas the conventional polymerization of S-alc gives water-insoluble products in a heterogeneous way. It was also confirmed that the reactivity and polymerization behavior of iso-S in the dehydrogenative polymerization are well reflected by those of S-alc. This unique polymerization system based on iso-S enabled us to follow the time-course of S-DHP formation in a homogeneous aqueous media by such as UV spectroscopy[26] and gel permeation chromatography with photodiode array detection (GPC-PDA).[27] Importantly, our approach has revealed that oligomeric S-QM accumulates stably during the HRP-catalyzed polymerization of iso-S. The low reactivity of S-QM can be explained by the presence of two electron-donating methoxyl groups, which reduce the positive charge density at the α-positions. It is reported that the analogous quinone methide, 2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone, also reacts very slowly in aqueous media. Bolton et al. pointed out that this low reactivity is caused by the lack of hydrogen bonding between the shielded oxo group and water molecules, suppressing charge separation of the quinone methide.[28,29] The same explanation can be applied for the low reactivity of S-QM.

The data in our previous studies strongly suggest that the low reactivity of S-QM with nucleophiles in the conventional polymerization system (at step 3 in Fig. 1) may retard the subsequent polymerization for S-DHP formation from S-alc. Based on this concept, if suitable nucleophiles with high nucleophilicity toward S-QM are added to the conventional polymerization system, they can perform nucleophilic additions to promote the rearomatization of S-QM, and the subsequent polymerization steps in Fig. 1 would then repeatedly proceed to yield S-DHPs efficiently. In the preliminary study, we showed that the HRP-catalyzed polymerization of S-alc in the presence of nucleophilic azide ion gave S-DHPs in significantly high yield.[30] In this article, further data on S-DHP formations in the presence of nucleophilic reagents are presented.

Experimental methods

Materials

Iso-S,[23] S-alc,[31] and syringylglycerol-β-syringyl ether [1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2,6-dimethoxyphenoxy)-propane-1,3-diol (**1**)][32,33] were synthesized according to the method described in the literature. HRP (100 U mg^{-1}) was purchased from Wako Pure Chemicals (Kyoto, Japan) and used without further purification. Wakogel C-200 (Wako Pure Chemicals) was used in silica gel column chromatography. Other chemicals were purchased from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemicals and used as received.

Screening of the nucleophile for HRP-catalyzed polymerization of S-alc by monitoring iso-S polymerization in the presence of nucleophilic reagents

UV spectroscopic monitoring of HRP-catalyzed polymerization of iso-S in the presence of nucleophiles was carried out as follows.[26] The solution (3 ml) consisting of 100 μM iso-S, 1500 μg l^{-1} HRP, and 100–1000 μM nucleophilic reagents (D-glucronic acid, ethyl amine, sodium sulfite, potassium iodide, cysteine, and sodium azide) in 50 mM sodium phosphate buffer (pH 6.5) and the same solution without monomers were placed in a sample cell and a reference cell, respectively. The cells were set in a JASCO V-560 spectrophotometer and kept at 25°C under stirring. The polymerization was initiated by adding 25 μl 0.024% hydrogen peroxide aqueous solution (final concentration, 60 μM) to the sample cell, and UV spectra were recorded at a regular time interval (scan rate, 2000 nm min^{-1}; scan region, 250–400 nm; data interval, 1 nm; response mode, quick).

GPC-PDA monitoring of the HRP-catalyzed polymerization of iso-S in the presence of azide ion was done as follows.[27] Three solutions were prepared for the polymerization of iso-S: solution A, 2.0 mg HRP in 500 μl 0.05 M

phosphate buffer (pH 6.5); solution B, 20 μmol glycosides in 2500 μl buffer; solution C, 2500 μl aqueous solution containing sodium azide (20 μmol) and hydrogen peroxide (24 μmol). Polymerization was initiated by adding solutions B and C simultaneously to solution A at a constant rate (2.5 ml h^{-1}; monomer addition time, 60 min). After initiating the polymerization, reaction mixtures (100 μl) were periodically sampled and mixed with 900 μl 0.1 M LiCl in dimethylformamide (DMF) to terminate the reaction, immediately cooled at 0°C, and subjected to GPC-PDA analysis within 15 min after withdrawing from the reaction mixture. The GPC-PDA analyses were performed on a Shimadzu LC-20A LC system (Shimadzu, Japan) equipped with a SPD-M20A photodiode array detector. Elution conditions were as follows: column, TSK gel α-M (Tosoh, Japan); eluent, 0.1 M LiCl in DMF; flow rate, 0.5 ml min^{-1}; column oven temperature, 40°C; injection volume, 20 μl. Conditions for PDA detection were as follows: scan region, 260–400 nm; bandwidth, 4 nm; response, 1280 ms. Molecular weight calibration was made using polystyrene standards (Shodex, Japan). Data acquisition and computation utilized LCsolution version 1.22 SP1 software (Shimadzu, Japan).

HRP-catalyzed polymerization of S-alc in the presence of azide ion

Two solutions were prepared for polymerization of S-alc: solution A, 120 ml distilled water containing 0.5 mmol S-alc and 3–12 mg HRP; and solution B, 120 ml 0.019% hydrogen peroxide (0.6 mmol) aqueous solution containing 0.5 mmol sodium azide. Solutions A and B were added dropwise to 30 ml 0.1 M phosphate buffer over a period of 0.5–48 h. The precipitate of the resulting polymer was collected by centrifugation (12000 rpm, 10 min), washed twice with distilled water, and lyophilized to obtain S-DHP.

S-DHPs were acetylated with standard protocols[15] and subjected to GPC and nuclear magnetic resonance (NMR) analyses. GPC was performed with a Shimadzu LC-10 system equipped with a UV-Vis detector (SPD-10Avp, monitoring at 280 nm) under the following conditions: columns, K-802, K-802.5, and K-805 (Shodex, Japan); eluent, $CHCl_3$; flow rate, 1.0 ml min^{-1}; column temperature, 40°C. The system was calibrated with polystyrene standards (Shodex). ^{1}H-, ^{13}C-, and two-dimensional (2D)-heteronuclear single quantum coherence (HSQC)-NMR spectra were collected with a Varian INOVA300 FT-NMR spectrometer (300 and 75.5 MHz for ^{1}H and ^{13}C nuclei, respectively) in chloroform-*d* with tetramethylsilane as the internal standard (0.0 ppm). Chemical shifts (δ) and coupling constants (J) were given in δ-values (ppm) and hertz (Hz), respectively.

3-Azido-3-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2,6-dimethoxyphenoxy)-1-propanol (**3**)

The quinone methide **2** was prepared from syringylglycerol-β-syringyl ether (**1**) by the method reported in the literature.[34,35] Briefly, compound **1** (380 mg, 1.0 mmol) was dissolved in 10 ml dichloromethane, and to this solution 260 μl trimethylsilyl bromide (2.0 mmol) was added with stirring under nitrogen at room temperature. After 1 min, the solution was poured into a separation funnel and extracted twice with 30 ml saturated sodium bicarbonate aqueous solution. The organic layer was dried over sodium sulfate and evaporated to dryness. The obtained reddish-colored solid of compound **2** was dissolved in 5 ml anhydrous dioxane and added dropwise into 4 ml dioxane/water solution (1:1, v/v) containing sodium azide (650 mg, 10 mmol) at 0°C under nitrogen. After 1 h, the reaction mixture was extracted with ethyl acetate, washed twice with saturated sodium chloride aqueous solution, and dried over sodium sulfate. Evaporation in vacuo produced an orange oil, which was purified by silica gel column chromatography [eluent, ethyl acetate/*n*-hexane (3:2, v/v)] to give compound **3** as a white solid (192.7 mg, 48% yield; *erythro*/*threo* = ~1.0). Stereochemical assignments were made from ^{1}H-NMR signals of propyl side-chain protons in analogy with the data of β-*O*-4 lignin model compounds in the literature.[36,37]

Acetate of compound **3**; ^{1}H-NMR (in $CDCl_3$): δ 1.96 (3H, s, C_γ-OCOCH_3, *erythro* isomer), 1.98 (3H, s, C_γ-OCOCH_3, *threo* isomer), 2.33 (3H, s, C_4-OCOCH_3), 3.77–3.82 (3.77, 3.80, 3.81, 3.82) (12H, s, Ar-OMe), 3.84–3.93 (1H, m, $H_{\gamma 1}$), 4.25–4.33 (1H, m, $H_{\gamma 2}$), 4.39–4.53 (1H, m, H_β), 4.91 (0.5H, d, J = 6.6 H_α, *erythro* isomer), 5.01 (1H, d, J = 4.8 H_α, *threo* isomer), 6.55 (2H, d, J = 3.0, $H_{2'}$ and $H_{6'}$, *threo* isomer), 6.58 (2H, d, J = 3.0, $H_{2'}$ and $H_{6'}$, *erythro* isomer), 6.65 (2H, s, H_2 and H_6, *threo* isomer), 6.71 (2H, s, H_2 and H_6, *erythro* isomer), 7.00 (1H, t, J = 8.7, $H_{1'}$, *erythro* isomer), 7.01 (1H, t, J = 8.7, $H_{1'}$, *threo* isomer). ^{13}C-NMR: δ 20.3, 20.6 (COCH_3), 55.8, 56.0, 56.1 (Ar-OMe), 62.7 (C_γ, *erythro* isomer), 63.1 (C_γ, *threo* isomer), 66.2 (C_α, *erythro* isomer), 66.7 (C_α, *threo* isomer), 81.2 (C_β, *erythro* isomer), 81.9 (C_β, *threo* isomer), 103.8 (C_2 and C_6, *threo* isomer), 104.2 (C_2 and C_6, *erythro* isomer), 104.6 ($C_{2'}$ and $C_{6'}$, *erythro* isomer), 104.9 ($C_{2'}$ and $C_{6'}$, *threo* isomer), 124.1 ($C_{1'}$), 128.1 (C_4, *erythro* isomer), 128.5 (C_4, *threo* isomer), 134.7 (C_1), 135.1 ($C_{4'}$, *erythro* isomer), 135.2 ($C_{4'}$, *threo* isomer), 151.9 (C_3 and C_5, *eryhtro* isomer), 152.0 (C_3 and C_5, *threo* isomer), 153.2 ($C_{3'}$ and $C_{5'}$), 168.5 (Ar-O*C*OCH$_3$), 170.3, 170.7 (C_γ-O*C*OCH$_3$)

Results and discussion

Screening of nucleophile for HRP-catalyzed polymerization of S-alc

In previous studies, we successfully detected and characterized S-QM formed in the HRP-catalyzed polymerization of iso-S using UV spectroscopic[26] and GPC-PDA[27] measurements. In the present study, these techniques were applied for screening of the nucleophile used as a S-QM scavenger in the polymerization of S-alc.

UV spectroscopic monitoring of HRP-catalyzed polymerization of iso-S in the presence of nucleophilic reagents

Figure 3A shows the time-dependent changes in UV spectra during the HRP-catalyzed polymerization of iso-S without nucleophiles. As the reaction time increased, the absorbance peak at 274 nm decreased, indicating that iso-S was oxidized by HRP. Formation and accumulation of stable S-QM were clearly indicated by the appearance of the absorption peak at 325 nm, as evidenced in our previous study.[26] A suitable nucleophile should not retard the HRP-catalyzed oxidation of iso-S, which can be evaluated by the decrease of the absorption at 274 nm (A_{274}), and the one should suppress the accumulation of S-QM, which can be evaluated by the increase of absorptions at 325 nm (A_{325}). Representative nucleophilic reagents investigated here are carboxyl acid (D-glucronic acid), amine (ethyl amine), sulfite ion (sodium sulfite), iodide ion (potassium iodide), thiol (cysteine), and azide ion (sodium azide). Figure 4 displays plots of A_{274} and A_{325} during iso-S polymerizations in the presence of the nucleophilic reagents. In polymerization with carboxylic acid, amine, sulfite ion, and iodide ion, A_{274} decreased smoothly, but A_{325} increased significantly, indicating high levels of S-QM accumulation (Fig. 4B–E). These results indicate that the nucleophilicity of these compounds toward S-QM is not sufficient under the present conditions. In several reports, highly nucleophilic thiol compounds[38–40] and azide ion[41–43] were used to trap QM species formed as reactive intermediates in various chemical reactions. On the other hand, both these are well-known peroxidase inhibitors.[44] When iso-S polymerization was conducted in the presence of cysteine (Fig. 4F), the decrease of A_{274} was much slower at the initial stage of polymerization (~30 min), while the increase in A_{325} was suppressed in this period. After a period of reaction time, A_{274} suddenly dropped and then A_{325} started increasing. Thiol compounds are reported to be substrates for HRP.[45,46] The result obtained here can be explained by the fact that HRP-catalyzed oxidation of cysteine took place in advance of the

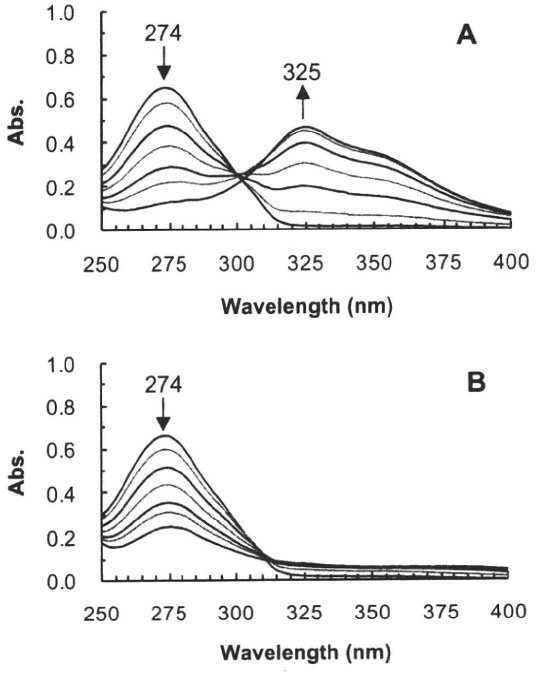

Fig. 3. UV spectra of polymerization mixtures during horseradish peroxidase (HRP)-catalyzed polymerizations of isosyringin (iso-S). **A** In the absence of nucleophiles (reaction time: 0, 2, 6, 10, 16, 20, and 30 min). **B** In the presence of azide ion (1 eq for iso-S): reaction time: 0, 2, 6, 10, 16, 10, 20, and 30 min. *Abs.*, absorbance

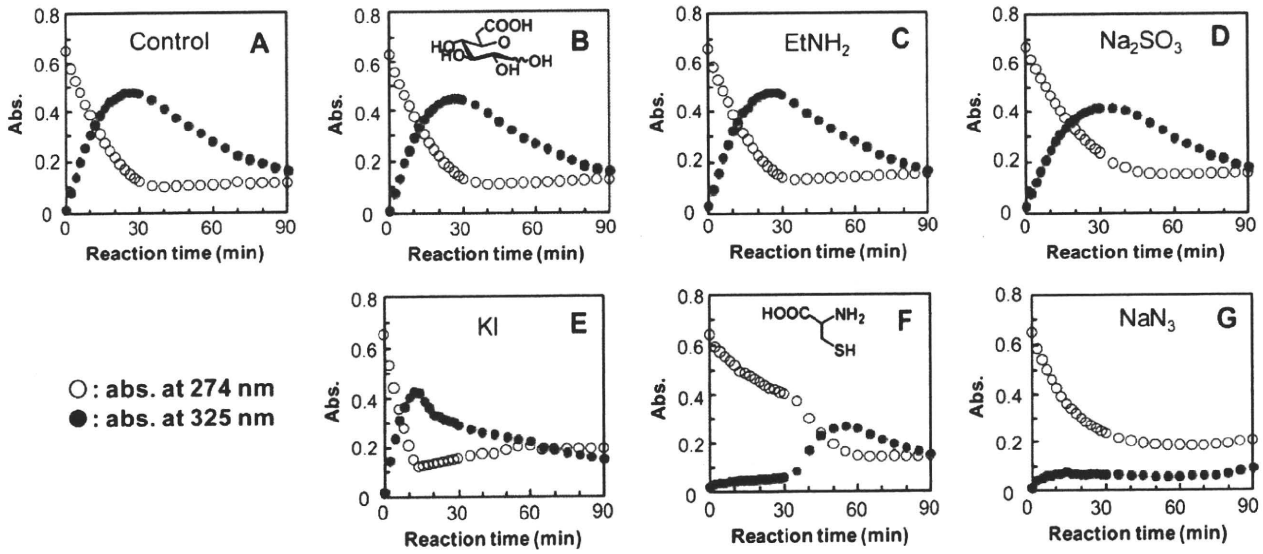

Fig. 4. Changes of absorbance at 274 nm (A_{274}, ○) and at 344 nm (A_{325}, ●) during HRP-catalyzed polymerizations of iso-S (100 μM) in the presence of various nucleophilic reagents: none (control) (**A**); D-glucronic acid (1000 μM) (**B**); ethyl amine ($EtNH_2$, 1000 μM) (**C**); sodium sulfite ($NaSO_3$, 500 μM) (**D**); potassium iodide (KI, 1000 μM) (**E**); cysteine (100 μM) (**F**); sodium azide (NaN_3, 100 μM) (**G**)

oxidation of iso-S. Thus, thiol compounds seem to be unsuitable as a S-QM scavenger used in HRP-catalyzed polymerization. On the other hand, A_{274} decreased smoothly in the presence of sodium azide, whereas the increase in A_{325} was hardly observed (Figs. 3B, 4G). The results indicated that azide ion efficiently scavenges S-QM without significant inhibition of the catalytic ability of HRP. Therefore, azide ion was concluded to be the most suitable nucleophile as a S-QM scavenger used in HRP-catalyzed polymerization of S-alc.

GPC-PDA monitoring of HRP-catalyzed polymerization of S-alc in the presence of azide ion

The HRP-catalyzed polymerization of iso-S in the presence of azide ion was monitored by GPC-PDA to obtain further confirmation of the ability of azide ion to trap S-QM. This method permits following the changes of the molecular weight of S-DHP intermediates as well as the formation of oligomeric S-QM during the course of the iso-S polymerization.[27] Figure 5 shows the GPC-PDA profiles of the iso-S polymerization in the absence and presence of azide ion. In the absence of azide ion, the presence of oligomeric S-QM was clearly indicated by the intense peak detected at 344 nm at 19.2 min of elution time (peak top MW = 1700) (Fig. 5A). As Fig. 5C shows, in the absence of azide ion, the peak area detected at 344 nm rose significantly just after initiating the polymerization and then decreased very slowly as reaction time progressed, indicating the transient but stable presence of oligomeric S-QM. In contrast, during polymerization in the presence of azide ion, the peak area from S-QM remained constantly low, indicating that accumulations of the oligomeric S-QM were effectively suppressed (Fig. 5B,C). This finding agrees well with the results in UV spectroscopic monitoring of the polymerization described above. Formation of polyphenolic S-DHP could be followed by absorption at 274 nm. The product molecular weights calculated based on PDA detection at 274 nm are plotted against reaction time in Fig. 5D. Clearly, the addition of azide ion to the polymerization system resulted in efficient formation of polyphenolic S-DHP, as the product molecular mass increased faster in polymerization with azide ion than without azide ion. These results are readily rationalized if the oligomeric S-QM are rapidly converted to the corresponding phenolics by azide addition and the resulting phenolics react further to produce S-DHP.

HRP-catalyzed polymerization of S-alc in the presence of azide ion

HRP-catalyzed polymerization of S-alc in the presence of azide ion, which serves as a S-QM scavenger, was carried out under various polymerization conditions. Figure 6 shows the effect of azide ion on the yield of isolated S-DHPs. The yield of S-DHPs prepared according to the so-called bulk polymerization method,[2] in which the monomer is added to the polymerization system dropwise but rapidly in 0.5 h, was much affected by the amount of sodium azide added to the polymerization system (Fig. 6A). As expected from earlier studies,[9-15] in the absence of azide ion, the yield of S-DHP was quite low (~5%). As the amount of sodium azide was increased to 1 eq for S-alc, the yield of S-DHP

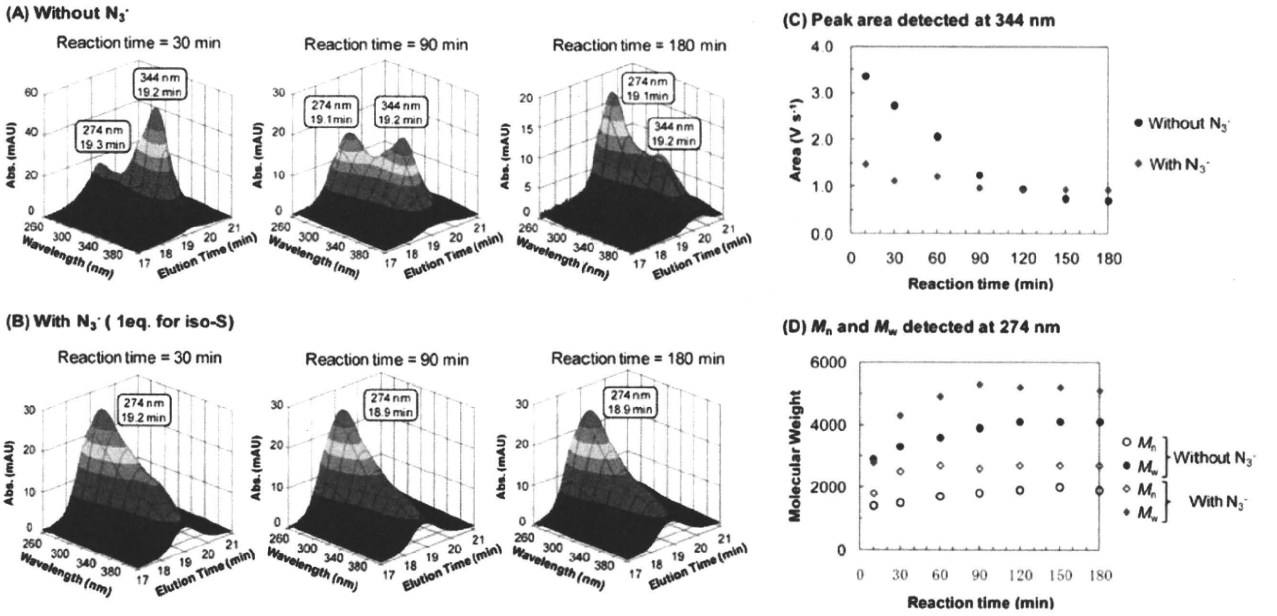

Fig. 5. Gel permeation chromatography with photodiode array detection (GPC-PDA) monitoring of HRP-catalyzed polymerization of iso-S. **A** Three-dimensional (3D) PDA plots in polymerization without nucleophilic regents. **B** 3D PDA plots in polymerization with azide ion (1 eq for iso-S). **C** Plots of peak area detected at 344 nm over reaction time. **D** Plots of number and weight average molecular weights (M_n and M_w) calculated based on PDA detection at 274 nm over reaction time

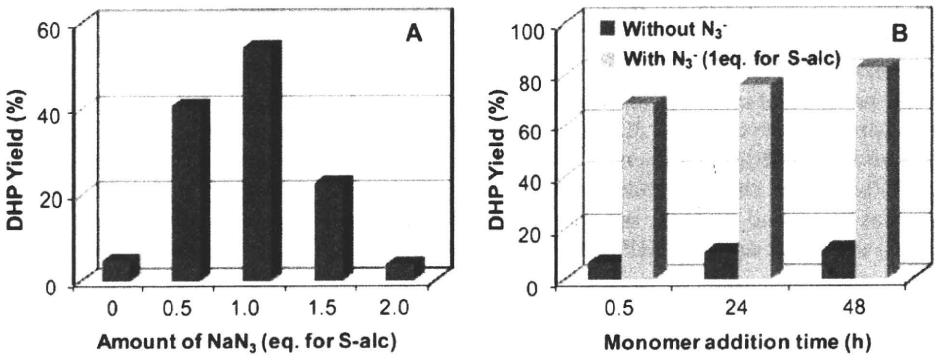

Fig. 6. Yields of syringyl dehydrogenation polymers (S-DHPs) in the HRP-catalyzed polymerization of S-alc in the presence of azide ion. **A** Effect of the amount of sodium azide (HRP = 6 mg for 1 mmol S-alc; monomer addition time = 0.5 h). **B** Effect of the monomer addition time (HRP = 24 mg for 1 mmol S-alc; sodium azide = 1 eq. for S-alc)

Table 1. HRP-catalyzed polymerizations of sinapyl alcohol (S-alc) in the presence and the absence of sodium azide

Entry	HRP[a] (mg)	Monomer addition time (h)	Without N_3^-				With N_3^- (1 eq for S-alc)			
			Yield (%)	M_n[b] × 10^{-3}	M_w/M_n[b]	DP_n[c]	Yield (%)	M_n[b] × 10^{-3}	M_w/M_n[b]	DP_n[c]
1	6	0.5	4.8	1.4	2.5	4.8	54.2	1.3	1.3	4.4
2	24	0.5	7.0	1.8	2.1	6.1	68.3	1.4	1.3	4.8
3	24	24	10.8	2.1	1.9	7.1	76.0	1.6	1.2	5.4
4	24	48	11.5	2.1	2.0	7.1	82.5	1.8	1.2	6.1

HRP. horseradish peroxidase
[a] Per 1 mmol of S-alc
[b] Determined by gel permeation chromatography (GPC) after acetylation
[c] Calculated based on the molecular weight of sinapyl alcohol diacetate

greatly increased to 54%. When an excess amount of sodium azide for S-alc was applied, however, the yield of S-DHP dropped again, probably because of inactivation of HRP induced by azide ion. Then, the so-called endwise polymerization method,[2] in which the monomer is added to the polymerization system slowly for 24–48 h, was employed with 1 eq sodium azide for S-alc. Figure 6B shows the effect of monomer addition time on the yield of S-DHP. The yield of S-DHP with azide ion further increased to 83% as the monomer addition time increased to 48 h, while the yield of S-DHP without azide ion also increased, but to no more than 12%. Table 1 lists the average molecular weights (M_n and M_w) and their distributions (M_w/M_n) of S-DHPs. The M_n values of the acetylated samples of S-DHPs prepared with azide ion were 1300–1800 (degree of polymerization, DP = 4–6), which are in the same range as those reported for the conventional DHPs.[47,48] The endwise polymerization method contributed to an increase in the molecular mass of S-DHP. It was observed that M_n and M_w/M_n values of the isolated S-DHPs prepared with azide ion were slightly lower than those for S-DHPs prepared without azide ion. This result may be explained by structural differences between them, as discussed in the next section. Nevertheless, it is obvious that an appropriate amount of azide ion (1 eq for S-alc) significantly promotes the production of S-DHP, indicating that the low reactivity of S-QM with nucleophiles is critically responsible for the low yield of S-DHP in the conventional polymerization system.

Structural characterization of S-DHPs

The ^{1}H-, ^{13}C-, and 2D-HSQC NMR spectra of acetylated S-DHPs prepared in the absence and presence of azide ion (prepared based on Table 1, entry 3) are shown in Fig. 7. Nucleophilic attacks of azide ion to S-QM during the polymerization are clearly demonstrated by the appearance of the signals from *β-O*-4/*α*-N_3 structure (**I**), which are identical to the data for *α*-azide model compound **3** synthesized according to Fig. 8. All the spectra indicate that the contributions from *β-O*-4/*α*-OH (**II**) and *β-O*-4/*α*-ether substructures (**III**) are negligibly small for S-DHP obtained with azide ion, whereas both structures are abundant for the conventional S-DHP prepared without azide ion. This result suggests that during the polymerization of S-alc with nucleophilic azide ion, the *β-O*-4 S-QM are exclusively quenched by azide ion but not by water, phenolic, or aliphatic hydroxyl groups. A series of peaks from *β-β* resinol structure (**IV**) is also observed in the spectra of S-DHP obtained with azide ion, indicating that *β-β* S-QM are rapidly trapped by intramolecular *γ*-hydroxyl groups, even in the presence of azide ion. Our preliminary data of Fourier transform-infrared (FT-IR) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses of S-DHPs also support this result.[30] As expected from these data, S-DHP prepared in the presence of azide ion is a simple linear polymer made up mainly of structure **I** and **IV**. As already mentioned, the S-DHPs prepared with azide