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RAPID COMMUNICATION

Yuki Tobimatsu · Toshiyuki Takano
Hiroshi Kamitakahara · Fumiaki Nakatsubo

Azide ion as a quinone methide scavenger in the horseradish peroxidase-catalyzed polymerization of sinapyl alcohol

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Key words Azide ion · Dehydrogenation polymer (DHP) · Horseradish peroxidase (HRP) · Quinone methide · Sinapyl alcohol

Introduction

The enzymatic dehydrogenative polymerizations of monolignols, mainly using horseradish peroxidase (HRP)/hydrogen peroxide as a catalytic system, has been widely used as a model to study lignification, yet there still remain several points to be clarified. One of them is the peculiar behavior of sinapyl alcohol (SA) in the in vitro polymerizations. Many researchers have reported that enzymatic dehydrogenative polymerizations of SA afforded synthetic lignin (dehydrogenation polymer: DHP), but in extremely low yields, whereas the polymerizations of coniferyl alcohol (CA) or *p*-coumaryl alcohol (PA) gave their DHPs in high yields.[1–4]

Enzymatic dehydrogenative polymerizations of monolignols proceed mainly by three steps: (1) radical formation by enzymatic dehydrogenation of phenolic hydroxyl groups; (2) radical coupling; (3) nucleophilic addition of nucleophiles to the quinone methide intermediates (QMs), resulting in the reproduction of phenolic compounds for subsequent oxidative couplings. The polymerization of SA is shown in Figure 1. Until now, the problems regarding the polymerizations of SA have been mainly discussed in terms of the reaction steps (1) and (2): the low reactivity of HRP to SA[5–7] and preferential β-β coupling reactions to β-*O*-4.[7,8] However, there is no report focused on reaction step (3) concerning the low polymerizability of SA.

Brunow et al.[9] reported that syringyl-type (S-type) quinone methide model compound was significantly less reactive than the guaiacyl type (G-type) in the reactions with water in aqueous dioxane. On the other hand, we recently detected the temporary presence of relatively stable S-type QMs during the HRP-catalyzed polymerizations of SA, and, analogously, of isosyringin (sinapyl alcohol γ-*O*-β-D-glucoside),[10] in phosphate buffer (pH = 6.5) (unpublished data). These observations suggest that the low yield of DHP from SA is probably due to the low reactivity of the S-type QMs.

Therefore, if suitable nucleophiles with higher nucleophilicity than water, phenolic, and aliphatic hydroxyl groups, were added to the polymerization system, they could perform nucleophilic additions to convert the QMs to corresponding phenolics. Then the three reaction steps shown in Figure 1 may repeatedly proceed to yield the DHP with a higher molecular weight in a high yield.

In this study, HRP-catalyzed polymerizations of SA were carried out in the presence of azide ion as a QM scavenger to prove the above presumption. This is the first report to describe the high-yield DHP production from SA in the presence of a nucleophile.

Results and discussion

Our preliminary experiments by ultraviolet-visible (UV-Vis) spectroscopy on the reactivities of the S-type QMs with various nucleophiles such as thiols, azide ion, iodide ion, amines, carboxylic acids, and so on, revealed that only thiols and azide ion immediately react with the QMs. However, most of the thiol compounds such as cysteine and its analogs were actually oxidized under the HRP-catalyzed reactions to afford complex products as reported.[11] Therefore, we selected azide ion as a nucleophile although it is well known that azide ion is a peroxidase inhibitor.[12]

The HRP-catalyzed polymerizations of SA were carried out in the presence of sodium azide according to the so-called bulk polymerization method[13,14] as follows. Two solutions were prepared for the polymerization: solution A consisted of 52.6 mg (0.25 mmol) of SA and 3 mg of HRP

Y. Tobimatsu (✉) · T. Takano · H. Kamitakahara · F. Nakatsubo
Division of Forest and Biomaterials Science, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan
Tel. +81-75-753-6255; Fax +81-75-753-6300
e-mail: tobikko@kais.kyoto-u.ac.jp

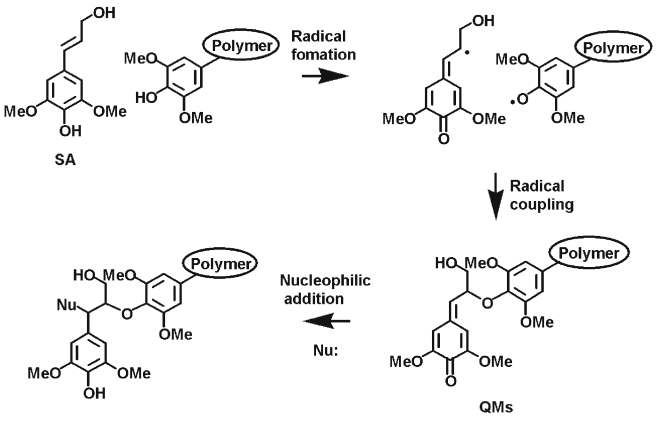

Fig. 1. Enzymatic dehydrogenative polymerization of sinapyl alcohol (*SA*)

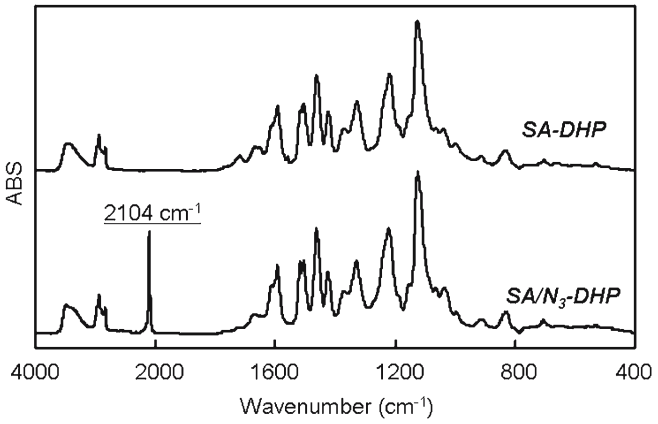

Fig. 3. Fourier transform infrared (FT-IR) spectrum of DHP prepared in the presence (*SA/N₃-DHP*) and absence (*SA-DHP*) of sodium azide (KBr method)

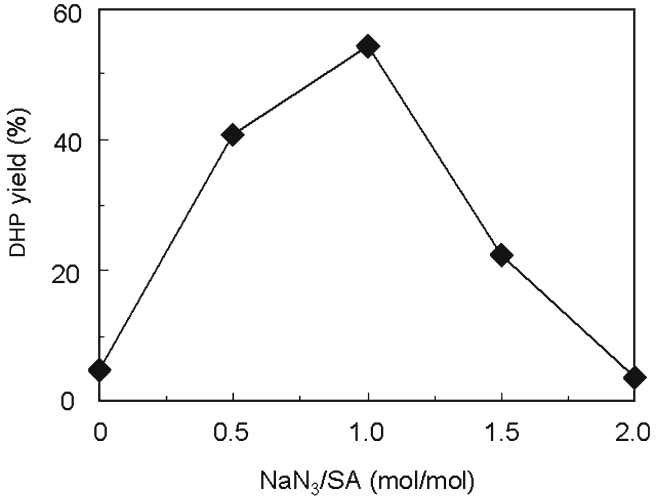

Fig. 2. Effect of the molar ratio of sodium azide to sinapyl alcohol (*NaN_3/SA*) on the yield of dehydrogenation polymer (*DHP*)

(100 U mg^{-1}, Wako) dissolved in 60 ml of distilled water; solution B consisted of 0–32.5 mg (0–0.5 mmol) of sodium azide dissolved in 60 ml of 0.019% hydrogen peroxide (0.3 mmol). Solutions A and B were gradually added to 15 ml of sodium phosphate buffer (0.1 M, pH 6.5) over 30 min at 25°C and allowed to stand for 24 h. The precipitates of the resulting DHP were collected by centrifugation and washed with distilled water and lyophilized.

As Fig. 2 shows, the yield of DHP was much affected by the molar ratios of sodium azide to SA (NaN_3/SA). In the absence of sodium azide (NaN_3/SA = 0), DHP was obtained in only 4.8% yield with orange color formation, as expected from earlier reports.[1,2] The yield of the DHP was greatly increased for increased NaN_3/SA, and then gradually decreased to 3.8% for NaN_3/SA = 2.0, indicating inactivation of HRP induced by azide ion. The maximum yield of DHP was 54.2% with NaN_3/SA = 1.0. Thus, it was found that the addition of an appropriate amount of sodium azide to the conventional polymerization system contributes to a remarkable increase in the yield of DHP from SA, as expected.

The structures of DHPs obtained by the polymerizations for NaN_3/SA = 1.0 (SA/N_3-DHP) and NaN_3/SA = 0 (SA-DHP) were characterized by Fourier transform infrared (FT-IR) spectroscopy, ^{1}H nuclear magnetic resonance (NMR) spectroscopy, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and gel permeation chromatography (GPC). For the ^{1}H NMR and GPC analyses, DHPs were acetylated with acetic anhydride and pyridine.[14,15]

The presence of azide group in SA/N_3-DHP was clearly proved by a strong absorption at 2104 cm^{-1} assigned to the stretching vibration of N≡N in the FT-IR spectrum (Fig. 3). In the ^{1}H NMR spectrum of acetylated SA/N_3-DHP, the peaks from the *β-β* resinol structure (3.1, 3.9, 4.3, and 4.7 ppm from the β, γ_1, γ_2, and α positions, respectively)[2] were observed, as well as being observed in the spectrum of acetylated SA-DHP (Fig. 4). This indicated that *β-β* QMs were preferentially attacked by intramolecular *γ*-hydroxyl groups, even in the presence of azide ion. On the other hand, the peaks from *α*-protons with *α*-OAc (6.0 ppm) and *α*-ether (5.7 ppm) in *β-O*-4 substructures were negligible in the spectrum of acetylated SA/N_3-DHP. Based on two-dimensional NMR techniques, a set of peaks assigned to *β-O*-4/*α*-N_3 structure (4.3, 4.4, 4.5, and 5.0 ppm from the γ_1, γ_2, β, and α positions, respectively) were clearly observed. These results strongly indicate that *β-O*-4 QMs were exclusively attacked by azide ion during the polymerization of SA in the presence of sodium azide. The existence of *β-O*-4/*α*-N_3 structures was also confirmed by MALDI-TOF MS (Fig. 5). A series of peaks assigned to trimer {[(*β-O*-4/*α*-N_3)-resinol Na]$^+$: calculated 692.2, found 692.5}, tetramer {[(*β-O*-4/*α*-N_3)$_2$-resinol Na]$^+$: calculated 943.3, found 943.5}, and pentamer {[(*β-O*-4/*α*-N_3)$_3$-resinol Na]$^+$: calculated 1194.4, found 1194.5} were observed, although small contribution of syringaresinol to the nucleophilic additions to *β-O*-4 QMs was indicated by the peaks in the higher molecular mass region: hexamer {[(*β-O*-4/*α*-N_3)-(*β-O*-4/*α-O*-resinol)-resinol Na]$^+$: calculated 1318.5, found 1319.0}; heptamer

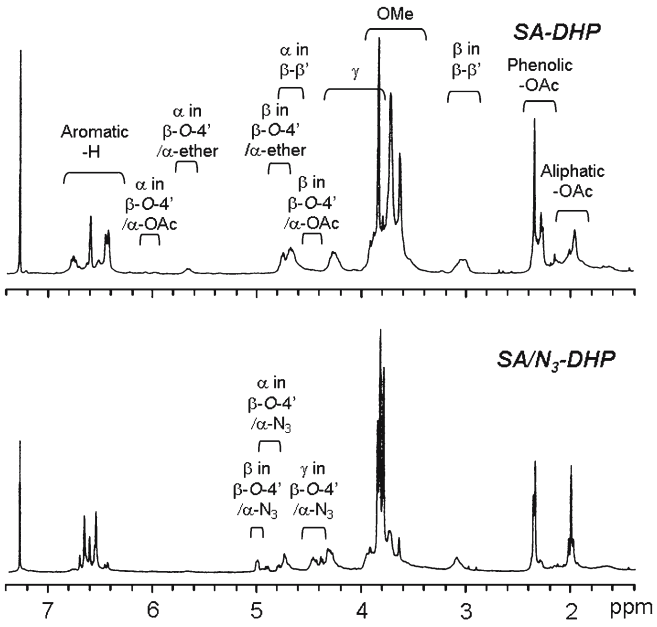

Fig. 4. ^{1}H Nuclear magnetic resonance (NMR) spectrum (300 MHz, $CDCl_3$) of acetylated samples of DHP prepared in the presence (*SA/N$_3$-DHP*) and absence (*SA-DHP*) of sodium azide

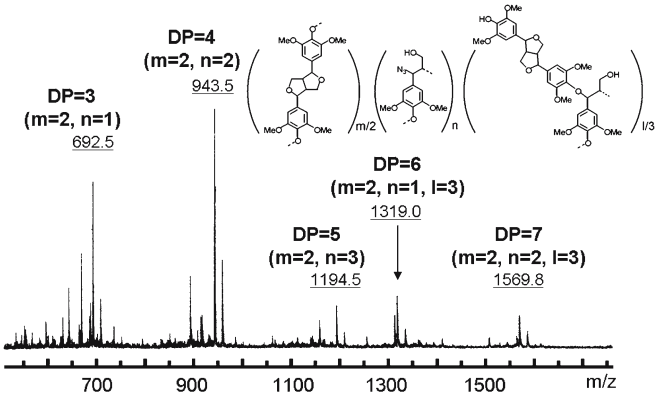

Fig. 5. Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrum of DHP prepared in the presence of sodium azide (SA/N$_3$-DHP) (positive reflection mode; 2,5-dihydroxybenzoic acid as matrix; DP: degree of polymerization)

{[(*β-O*-4/*α*-N$_3$)$_2$-(*β-O*-4/*α-O*-resinol)-resinol Na]$^+$: calculated 1569.6, found 1569.8}. Number-average molecular weights ($\bar{M}_n$) of acetylated SA/N$_3$-DHP and SA-DHP were estimated by GPC (polystyrene standards) to be 1300 and 1400, respectively: these values are almost in the same range as reported values[2,14,15] and were in reasonable agreement with the results of MALDI-TOF MS analysis.

Thus, these structural analyses clearly demonstrated that SA/N$_3$-DHP is a polymer produced mainly by repetitive reactions of monomer with oligomers via *β-O*-4 coupling followed by nucleophilic additions of azide ion to the QMs. As a consequence, it was indicated for the first time that in the HRP-catalyzed polymerization of SA in the presence of a strong nucleophile, the conversion of the unreactive S-type QMs to the corresponding phenolics is promoted, resulting in a great increase in the yield of DHP. Moreover, we expect a higher yield production of DHP with a higher $\bar{M}_n$ by so-called end-wise polymerization.[13,14]

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