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Enzymatic Polymerization of Coniferyl Alcohol in the Presence of Cyclodextrins

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The dehydrogenative polymerization of coniferyl alcohol by horseradish peroxidase was performed in 0.10 M phosphate buffer at 27 °C. Dehydrogenative polymer (DHP) from coniferyl alcohol was characterized by size exclusion chromatography (SEC) and nuclear magnetic resonance (NMR) spectroscopy. The ratio of 8-*O*-4′:8-5′:8-8′ linkages was determined by the ^{1}H NMR spectrum of DHP acetate which had good solubility. In "end-wise like" polymerization (the slow addition of hydrogen peroxide), addition of α-cyclodextrin to the medium led to DHP with increased 8-*O*-4′ content and a decrease in 8-5′ linkages. Under higher pH conditions, DHP with higher 8-*O*-4′ and 8-5′ content was obtained in the presence of α-cyclodextrin. In the end-wise polymerization (the slow additions of coniferyl alcohol and hydrogen peroxide), using α-cyclodextrin also gave DHP with a 8-*O*-4′ richer structure than that prepared in no additive system. The analysis of thioacidolysis products from DHP supported the results of the α-cyclodextrin effects on the 8-*O*-4′-rich structure of DHP. The 8-*O*-4′ structure in DHP prepared in the presence of α-cyclodextrin had racemic form as shown by ozonation.

Introduction

Enzymatic polymerization has received much attention as a new method for polymer synthesis to satisfy the increased demands for the production of various functional polymers.[1,2] The polymerization of phenolic compounds has been extensively investigated in recent decades by mimicking nature, in which various oxidoreductases play important roles in maintaining the metabolism of living systems.[3] Coniferyl alcohol is a well-known phenolic lignin monomer (monolignol) that is essential for lignin formation in the cell walls of woody plants (Figure 1).[4−6] In lignin biosynthesis, peroxidases catalyze the dehydrogenation of monolignols. Activated monolignols are randomly coupled, and the repeated coupling reaction leads to lignin with mixed linkages between the phenylpropane units (e.g., 8-*O*-4′, 8-5′, and 8-8′ structures; Figure 1). This irregular structure presents a barrier to the use of lignin in the field of polymeric materials. If regulation of the chemical structure of lignin could be achieved, the relationships between the chemical structure and the properties of lignin could be elucidated, giving important information for the development of novel functional materials derived from lignin.[7]

Controlled polymerization using constrained geometries is an important technique for the regulation of the chemical structure.[8] Micelles, mesoporous materials, polymer templates, Langmuir-Blodget films (LB films), and inclusion complexes have been used for the design of polymers having controlled structures. Polymerization of phenol compounds using a reversed micellar system[9] and mesoporous silica[10] was conducted to obtain polyphenols with a controlled structure. Kobayashi's group reported template polymerization of phenol derivatives using the interaction between the phenol derivatives and poly(ethylene glycol).[11] Lignin is biosynthesized in the presence of polysaccharide templates (i.e., cellulose and hemicellulose).[12] The polymerization of monolignol in vivo leads to a relatively controlled structure that is rich in the 8-*O*-4′ linkage. On the other hand, a dehydrogenative polymer (DHP) prepared in vitro has a random structure which is poor in the 8-*O*-4′ linkage.[13] For obtaining the DHP with natural-wood-like-lignin structure, the synthesis of DHP by using polysaccharide templates was previously reported.[14−18] Terashima et al. proposed that dehydrogenative polymerization of coniferyl alcohol was investigated in the presence of pectin as a polysaccharide matrix in a biomimetic system.[14] In their report, DHP with a 8-*O*-4′-rich structure was obtained under acidic conditions. Cathala's works provided that DHP with a higher molecular weight was obtained by using pectin.[16] They also reported morphological information about DHP prepared under the biomimetic conditions.[17]

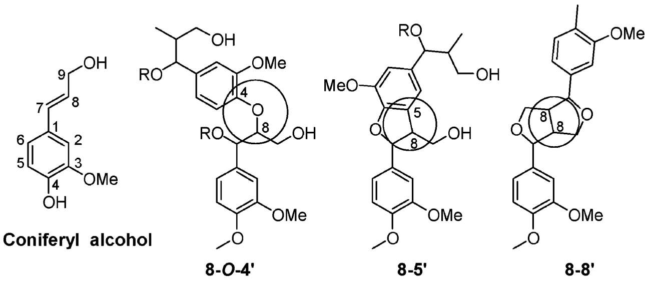

Figure 1. Structures of coniferyl alcohol and 8-*O*-4′, 8-5′, and 8-8′ linkages in lignin.

Cyclodextrins (CDs) have an inclusion ability for benzene derivatives, forming supramolecular structures.[19] The formation of inclusion complexes between CDs and phenols in the polymerization step probably regulates the structure of polymers. Some studies about the enzymatic polymerizations of phenol compounds by the use of peroxidases in the presence of CDs were previously reported.[20,21] Ritter et al. reported the enzymatic polymerization of aminochalcone (3-(4-aminophenyl)-2-propen-1-one) derivatives in the presence of 2,6-dimethyl-*β*-CD, in which the polymerized products were obtained with regulated

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Table 1. Dehydrogenative Polymerization of Coniferyl Alcohol by HRP/H_2O_2

			additive				product			
entry	method	monomer, mg		equiv	HRP, mg	pH	weight, mg	yield, %	$M_n \times 10^{3e}$	M_w/M_n^e
1	A[a]	100	none		0.22	7.4	84	84	2.1	1.8_3
2	A[a]	100	α-CD	1.5	0.22	7.4	77	71[d]	2.0	1.5_5
3	A[a]	100	α-CD	1.5	0.22	5.5	93	82[d]	1.4	1.3_0
4	A[a]	100	α-CD	1.5	0.22	9.0	79	72[d]	3.1	1.4_5
5	A[a]	100	β-CD	1.5	0.22	7.4	68	61[d]	2.9	1.5_3
6	B1[a]	100	none		0.22	7.4	86	86	2.5	1.6_0
7	B1[a]	100	α-CD	0.5	0.22	7.4	70	61[d]	3.3	1.4_2
8	B1[a]	100	α-CD	1.5	0.22	7.4	74	68[d]	2.5	1.5_1
9	B2[b]	100 × 3[c]	α-CD	1.5	0.22 × 3[c]	7.4	250	70[d]	2.9	1.5_2
10	B1[a]	100	α-CD	1.5	0.66	7.4	88	83[d]	2.6	1.6_1

[a] Reaction conditions: 100 mg of coniferyl alcohol (0.56 mmol), 4.0 mL of 0.50% H_2O_2 (0.67 mmol) solution, in 0.10 M phosphate buffer (pH 7.4), for 26 h at 27 °C. [b] Reaction conditions: slow addition of coniferyl alcohol/α-CD (100 mg 811 mg^{-1}, i.e., 0.56/0.84 mmol) thrice, 4.0 mL of 0.50% H_2O_2 (0.67 mmol) solution thrice, in 0.10 M phosphate buffer (pH 7.4), for 24 × 3 h (total 72 h) at 27 °C. [c] Additions of coniferyl alcohol/α-CD (100 mg 811 mg^{-1}, i.e., 0.56/0.84 mmol) and horseradish peroxidase (0.22 mg) every 24 h three times. [d] Yield (%) caluculated according to the following equation: yield (%) = [weight of the product (mg) − weight of sugar contained in the product (mg)]/coniferyl alcohol used in the polymerization (mg). [e] Estimated by SEC (polystyrene standards).

structures.[20] Kobayashi's group also showed the enzymatic polymerization of phenol and its derivatives by using CD derivatives.[21]

There is no report about the enzymatic polymerization of coniferyl alcohol using CDs. It is more difficult to regulate the structure of artificial lignin by the enzymatic polymerization of monolignol compared with the case of other phenol compounds, e.g., alkylphenols. In this study, to obtain 8-*O*-4′-rich lignin in vitro synthesis, the enzymatic polymerization of coniferyl alcohol was performed in the presence of CDs. The application of the enzymatic polymerization using CDs to the lignin synthesis is the first study, which probably leads to the regulation of lignin structure. The effect of the addition of CDs on the DHP structure is discussed in comparison with no additive system. The ratio of the main linkages 8-*O*-4′, 8-5′, and 8-8′, was determined by ^{1}H NMR of the DHP acetate. The interaction of coniferyl alcohol with a CD was also investigated by ^{1}H NMR measurement of a coniferyl alcohol/CD mixture. The 8-*O*-4′ structure of DHP was investigated in detail by thioacidolysis and ozonation. CD effect on the structure of DHP is also discussed.

Experimental Section

Material. Coniferyl alcohol was prepared according to the method of Ralph et al.[22] Horseradish peroxidase (HRP), α-cyclodextrin (α-CD), and β-cyclodextrin (β-CD) were purchased from Wako Chemical Co. (Osaka, Japan) and were used without further purification. Dimethyl sulfoxide-d_6 and chloroform-*d* for NMR were obtained from Aldrich Chemical (St. Louis, MI) and stored at 4 °C.

DHP Synthesis by Method A ("End-Wide Like" Polymerization). The general procedure is as follows (entry 2 in Table 1). Coniferyl alcohol (100 mg, i.e., 0.56 mmol), HRP (0.22 mg), and α-CD (811 mg, i.e., 0.84 mmol) were dissolved in 120 mL of 0.10 M phosphate buffer (pH 7.4). While vigorously stirring the mixture, 4.0 mL of 0.50% H_2O_2 (0.67 mmol) was added over 20 h using a micro pump. The polymerization was worked up by the addition of 3.0 mol/L hydrochloric acid until the reaction mixture was at pH 2−3. The mixture was centrifuged, and the supernatant was discarded. After the precipitate was collected, it was washed twice with distilled water. The residue was dispersed in distilled water, and the mixture was lyophilized to obtain a yellow powder. Percent yield was calculated as follows:

$$\%\ \text{yield} = [\text{weight of the product (mg)} - \text{weight of sugar contained in the product (mg)}]/\text{coniferyl alcohol used in the polymerization (mg)} \times 100$$

The weight of sugar content was determined by the operation of the product with phenol and sulfuric acid (97%). The experiment is mentioned later in detail.

We performed the polymerization at pH 5.5 and 9.0 as a similar manner at pH 7.4 except for using 0.10 M phosphate buffer at pH 5.5 and 9.0.

DHP Synthesis by Method B1 ("End-Wise" Polymerization; Type 1). The general procedure is as follows (entry 7 in Table 1). Coniferyl alcohol (0.56 mmol) and α-CD (0.84 mmol) were dissolved in 0.10 M phosphate buffer (pH 7.4, 10 mL). HRP (0.22 mg) was dissolved in another phosphate buffer (0.10 M, pH 7.4, 110 mL). While stirring, the solutions of α-CD/coniferyl alcohol and 4.0 mL of 0.50% hydrogen peroxide (0.67 mmol) were slowly added to the HRP solution over 20 h using micro pumps. The mixture was stirred for 6 h after completion of the addition. Distilled water was added to the solid after lyophylization of the reaction mixture, and the solution was adjusted to pH 2−3 using 3.0 mol/L hydrochloric acid. The precipitate was collected by centrifugation and washed twice with distilled water. The precipitate was dispersed in distilled water and then lyophylized to obtain the DHP. Calculation of percent yield was the same manner as method A.

DHP Synthesis by Method B2 ("End-Wise" Polymerization; Type 2; Entry 9 in Table 1). Coniferyl alcohol (0.56 mmol) and α-CD (0.84 mmol) were dissolved in 0.10 M phosphate buffer (pH 7.4, 10 mL). HRP (0.22 mg) was dissolved in another phosphate buffer (0.10 M, pH 7.4, 110 mL). While stirring, the solutions of α-CD/coniferyl alcohol and 4.0 mL of 0.50% hydrogen peroxide (0.67 mmol) were slowly added to the HRP solution over 20 h using micro pumps. The mixture was stirred for 4 h after completion of the addition. The slow additions of 10 mL of α-CD/coniferyl alcohol (0.56 mmol/0.84 mmol) and 4.0 mL of 0.50% hydrogen peroxide (0.67 mmol) for 20 h and stirring for 4 h were performed (a total of 3 dosings). A total of 0.22 mg of HRP was added every 24 h. Purification was conducted in a similar manner to that of method B1. Calculation of percent yield was done in the same manner as in method A.

Acetylation of DHP.[23] DHP (20 mg) was dissolved in 0.25 mL of pyridine. Acetic anhydride (0.25 mL) was added to the reaction mixture while stirring. After 24 h, the reaction was quenched by the addition of 0.10 mL of ethanol. DHP acetate was extracted using chloroform and recovered by evaporation of the chloroform. The remaining pyridine and acetic acid were removed by repeated coevaporation using ethanol (yield 14−22 mg).

Quantification of CD in the Product.[24] The product (ca. 0.5 mg) in DHP synthesis was dispersed in water (1.0 mL) and 80% phenol solution (0.025 mL). Sulufric acid (97%, 2.5 mL) was slowly added to the mixture at 10 °C. After keeping the solution at 20 °C for 10 min, the absorbance of the solution at 490 nm was measured by a UV/vis spectrometer.

Thioacidolysis and Subsequent Raney Nickel Desulfurization. Thioacidolysis and the subsequent Raney nickel desulfurization of DHP were conducted according to Lappierre et al.[25,26] The identities of

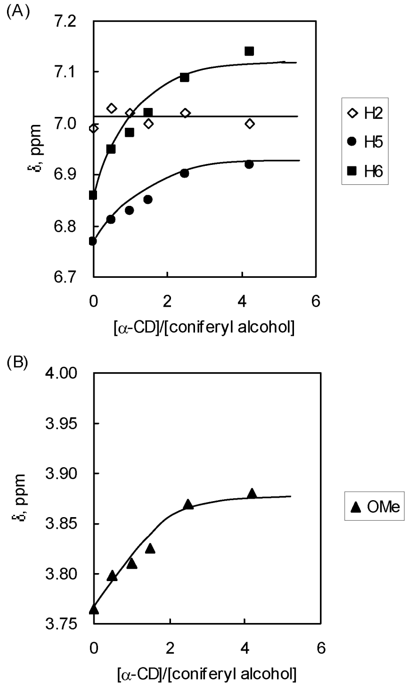


Figure 2. ^{1}H chemical shift displacements of the lower fields of aromatic resonances (A) and methoxy groups (B) in coniferyl alcohol on addition of α-CD in D_2O; [coniferyl alcohol] = 1.4 mg mL^{-1}.

various monomeric and dimeric degradation products were systematically verified by gas chromatography−mass spectroscopy (GC−MS).

Determination of the Ratio of *erythro* and *threo* Forms of 8-*O*-4′ Structures. The ratio of *erythro* to *threo* forms of the 8-*O*-4′ structures in DHP was determined by ozonation analysis according to the method of Akiyama.[27] The identities of erythronic acid and threonic acid generated from DHP by ozonation were verified by GC-MS.

Measurement. NMR spectra were recorded on a Bruker AMX 400 spectrometer with 16 scans for ^{1}H NMR and 20 000 scans for ^{13}C NMR measurements. DHP and DHP acetate were measured in DMSO-d_6 and chloroform-*d*, respectively (at 27 °C). ^{1}H NMR of the mixture of conifery alcohol and α-CD was performed in deuterium oxide (at 27 °C). Molecular weights and polydispersities were determined by size exclusion chromatography (SEC) on a Shimadzu SCL-10A apparatus equipped with a UV detector (monitoring at 280 nm) and TSK gel α-2500 column (Tosoh, Japan), eluted with DMF containing 0.10 M LiCl at a flow rate of 0.5 mL min^{-1} at 60 °C (using poly(styrene) as standards). Absorbance at 490 nm was measured by a JASCO V-530 UV/vis spectrophotometer. GC−MS spectra were recorded on Shimadzu GC-2010 and GCMS-Q2010 apparatus equipped with DB-1 columns (Agilent Technologies, USA). Ozonation was performed using a QOT-31R-2 ozone generator (Nippon Ozone Co., Ltd. Japan).

Results and Discussion

Inclusion Complex of Coniferyl Alcohol with CD. It is known that CDs and their derivatives have inclusion ability for benzene derivatives in aqueous solution. To evaluate the intermolecular interaction of coniferyl alcohol with α-CD, ^{1}H NMR of the mixture of coniferyl alcohol and α-CD was analyzed. The chemical shift of aromatic ring protons derived from coniferyl alcohol was changed by the addition of α-CD (Figure 2A). The signals derived from H_5 and H_6 in the aromatic ring in coniferyl alcohol were shifted to lower fields with an increasing amount of α-CD. The signal of the methoxy group also shifted to lower fields with an increasing amount of α-CD (Figure 2B). The H_5, H_6, and Hs of the methoxy group were close to the pyranose rings of α-CD, which interacted with α-CD (Figure 3). In contrast, no change was observed in the signal of H_2, which indicates no interaction between H_2 and α-CD (Figure 3). This is probably because of the greater distance between the H_2 of coniferyl alcohol and the pyranose rings caused by the presence of the methoxy group at the C_3 position. The signals of H_7, H_8, and H_9 have also shown no change by adding α-CD. The observations indicate that CD only interacts with an aromatic ring of coniferyl alcohol. Coniferyl alcohol forms a complete inclusion complex with α-CD with 1.4 equiv of α-CD for each coniferyl alcohol.[28] This shows that the inclusion of coniferyl alcohol in α-CD is an equilibrium process with a relatively weak interaction between coniferyl alcohol and α-CD.

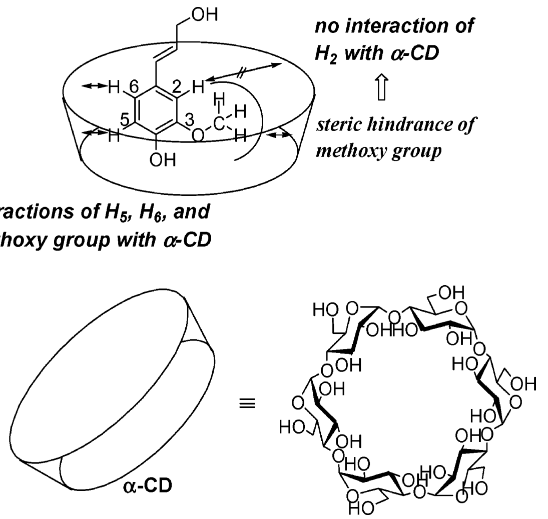


Figure 3. Morphological information of the inclusion complex of α-CD with coniferyl alcohol in aqueous solution estimated by ^{1}H NMR.

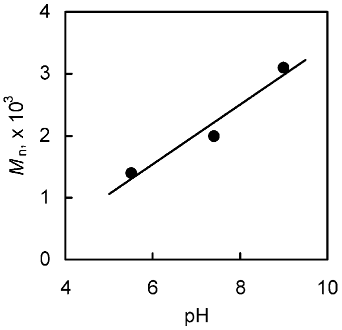


Figure 4. Number-average molecular weight of DHP prepared by method A under various pH conditions using α-CD. Preparations were in 0.10 M phosphate buffer at 27 °C.

DHP Synthesis. Enzymatic polymerization of coniferyl alcohol using horseradish peroxidase (HRP)/H_2O_2 was performed in 0.10 M phosphate buffer at 27 °C. In entries 1−5 in Table 1, DHP was synthesized by method A, which slows the addition of 0.50% H_2O_2 to the dehydrogenation system including coniferyl alcohol, a CD, and HRP. In the no additive system, DHP was obtained in good yield (84%; Table 1, entry 1). In contrast to the no additive system (entry 1), DHP prepared in the presence of α-CD gave a relatively lower yield (71%; Table 1, entry 2). Figure 4 illustrates the relationship of the molecular weight of DHP versus the pH of the reaction medium. Addition of α-CD had no effect on the molecular weight of DHP. On the other hand, using *β*-CD as an additive gave DHP with a slightly higher molecular weight than that prepared in the no additive system (entry 5). In the α-CD system, an increase in the molecular weight was observed at higher pH, and DHP was

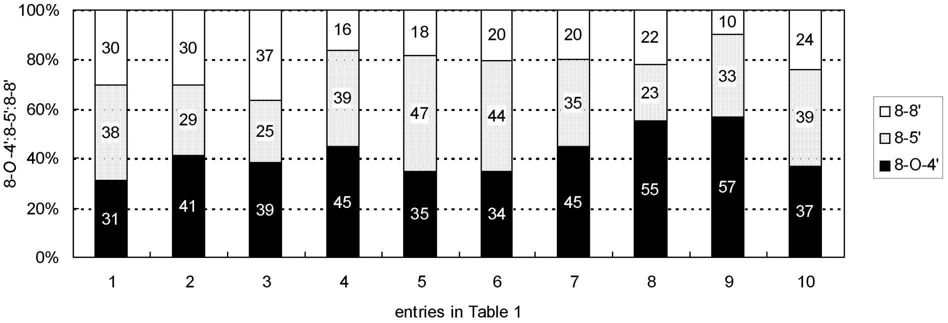


Figure 5. 8-*O*-4′:8-5′:8-8′ ratio of DHP determined by ^{1}H NMR of DHP acetate in $CDCl_3$ at 27 °C (entry numbers correspond to those in Table 1).

more soluble in phosphate buffer (pH $\geq$ 7.4) because of the formation of phenolic anions in the buffer (entries 2−4). This improved solubility of DHP probably contributed to the increase in molecular weight. Figure 5 shows the 8-*O*-4′:8-5′:8-8′ ratio of DHP. The 8-*O*-4′:8-5′:8-8′ ratio was determined from the ^{1}H NMR spectrum of DHP acetate. The characteristic signals of each linkage appeared at 4.81 (derived from H_8 of 8-*O*-4′ structure), 5.43 (derived from H_7 of 8-5′ structure), and 3.10 ppm (derived from H_8 of 8-8′ structure). The 8-*O*-4′:8-5′:8-8′ ratio was calculated using these signals. When DHP was prepared using the no additive system and α-CD system, the ratios of 8-*O*-4′:8-5′:8-8′ were 31:38:30 and 41:29:30, respectively. This result indicated that the effect of α-CD is the increase of 8-*O*-4′ content with the decrease of 8-5′ content. The 8-8′ content was not effected by the addition of α-CD. An increase in the 8-5′ content and a decrease in the 8-8′ content were observed at higher pH condition. The 8-*O*-4′ content was slightly increased with a higher pH. On the other hand, when DHP was prepared using *β*-CD, the ratio of 8-*O*-4′:8-5′:8-8′ was 35:47:18. Addition of *β*-CD did not give DHP with a 8-*O*-4′-rich structure (entry 5). The difference in the observation concerning 8-*O*-4′ content was attributed to the cavity size of CDs.

The end-wise polymerization (methods B1 and B2) involves slow addition of monomer and catalyst. The end-wise polymerization of coniferyl alcohol is an example of a biomimetic method of lignin biosynthesis, in which coniferyl alcohol is supplied slowly. This technique leads to DHP with a controlled structure rich in 8-*O*-4′ linkages.[13] The enzymatic polymerization of coniferyl alcohol using HRP/H_2O_2 by the end-wise method was conducted in 0.10 M phosphate buffer (pH 7.4) at 27 °C. Entries 6−10 in Table 1 show the result of DHP synthesis by method B. The number-average molecular weight of DHP prepared in the conventional end-wise polymerization was 2500, which was similar to method A (entry 6). Using a small amount of α-CD (0.5 equivalents for coniferyl alcohol) led to DHP with a relatively higher molecular weight (entry 7). However, the molecular weight decreased with an increase in α-CD (1.5 equiv for coniferyl alcohol; entry 8). The decrease in molecular weight was attributed to the formation of an inclusion complex in the monomer. The addition of a small amount of α-CD (0.5 equiv for coniferyl alcohol) gave the improvement of solubility of DHP, which led to higher molecular weight. However, an increase in α-CD (1.5 equiv for coniferyl alcohol) caused the steric hindrance derived from the inclusion complex to polymerize coniferyl alcohol, which gave DHP with lower molecular weight. Entries 6−10 in Figure 5 show the ratio of the structures under various conditions of end-wise polymerization. As shown in entry 6 of Figure 5, The ratio of 34:44:20 (8-*O*-4′:8-5′:8-8′) indicated that DHP prepared in the conventional end-wise method had a random structure. Increasing α-CD led to DHP with 8-*O*-4′-rich and 8-5′-poor structures (entries 7 and 8). The content of 8-8′ varied little with the amount of α-CD. The 8-*O*-4′:8-5′:8-8′ ratio of DHP prepared in α-CD (1.5 equiv for coniferyl alcohol) as an additive was 55:23:22, which led to DHP with an 8-*O*-4′-richer structure (entry 8). This controlled structure of DHP was attributed to the inclusion complex of coniferyl alcohol with α-CD in the propagation step. The tendency of DHP toward 8-*O*-4′-rich structures was in agreement with that prepared by method A. Prolonged addition of coniferyl alcohol with the addition of HRP every 24 h (a total of 3 dosings) led to a slight increase in the molecular weight of the DHP (from 2500 to 2900), in which the 8-*O*-4′-rich structure was retained (entry 9). On the other hand, adding 3-times the amount of HRP at the start produced DHP with a random structure when it was prepared in α-CD (1.5 equiv for coniferyl alcohol; entry 10).

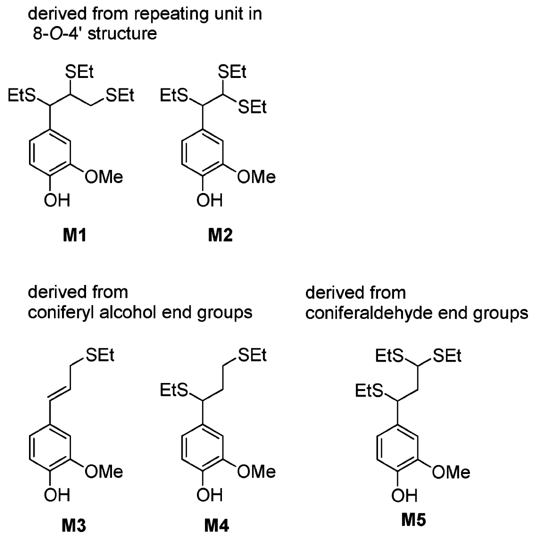


Figure 6. Structure of monomer units obtained by thioacidolysis of DHP.

When a lignin sample is subjected to thioacidolysis (solvolysis in dioxane−ethanethiol with boron trifluoride etherate), the 8-*O*-4′ bonds are efficiently cleaved without attendant condensation reactions that form new C−C linkages. The quantification of the main degradation monomer gives a close approximation of the phenylpropane units linked by 8-*O*-4′ bonds. Figure 6 illustrates the thioethylated monomers, and Table 2 shows the distribution of monomeric thioacidolysis products. Among the thioethylated monomers shown, the compounds **M1** (corresponding peak in GC−MS at 35.4 and 35.6 min) and **M2** (22.2 min) are derived from 8-*O*-4′-linked repeating units, and **M3** (18.4 min) and **M4** (25.0 min) are derived from coniferyl alcohol end groups (Figure 6). **M5**, obtained from coniferaldehyde end groups, was not observed in the thioacidolysis. The entry

Table 2. Yields of Thioethylated Monomer Recovered from Thioacidolysis and Raney Nickel Desulfurization of Thioethylated Dimer Recovered from Thioacidolysis of DHP in Table 1

	thioacidolysis			Raney nickel desulfurization	
entry	M1 + M2, mmol g^{-1}	M3 + M4, mmol g^{-1}	total yield, mmol g^{-1}	5-5′ mol %	total yield mmol g^{-1}
6	0.59	0.035	0.62	45	0.032
7	0.59	0.060	0.65	31	0.041
8	1.01	0.19	1.19	25	0.124
9	1.03	0.082	1.11	21	0.213
10	0.50	0.14	0.62	40	0.119

numbers in Table 2 correspond with DHP prepared under the conditions in Table 1. The total yield of monomers reflects the amount of phenylpropane units involved only in 8-*O*-4′ linkages. The yields of thioethylated monomers obtained from DHP prepared in the α-CD system were higher than that in the no additive system (entries 6−8). This indicates that DHP prepared in α-CD had a higher 8-*O*-4′ linkage content than in the no additive system, which agreed with the estimation by ^{1}H NMR (Figure 7). For DHP in entries 9 and 10, the estimation of the 8-*O*-4′ linkage content by thioacidolysis was also in agreement with results from ^{1}H NMR.

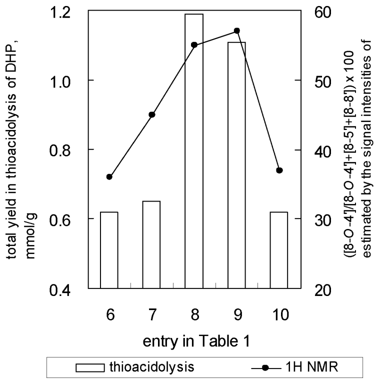
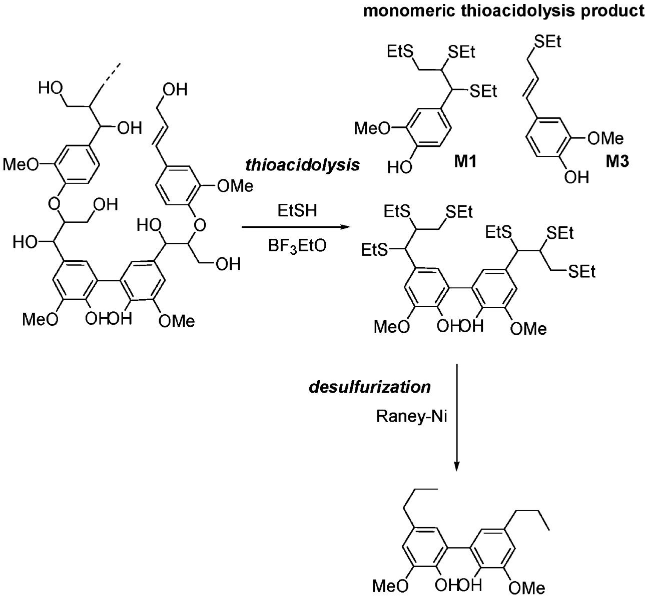


Figure 7. Relationship between the total yield of monomer units by the thioacidolysis of DHP and the 8-*O*-4′ ratio estimated by ^{1}H NMR.

The desulfurization of thioacidolysate with Raney nickel gives information on the condensed linkages as dimeric structures (Scheme 1). The results of analysis of the dimeric structures, which were identified by GC−MS, are summarized in Table 2. The total yield of dimers reflects the quantity of 8-*O*-4′ linkages. Higher dimmer yields are caused by a higher content of dimers connected by 8-*O*-4′ linkages. With increased α-CD in the DHP production, a higher total yield of recovered dimers was observed (entries 6−8). This observation supported the results of ^{1}H NMR and thioacidolysis, in which addition of α-CD led to DHP with an 8-*O*-4′-rich structure. The 8-*O*-4′ linkage cleaved by thioacidolysis gave a higher yield by desulfurization. The relative yield (mol %) of the 5-5′ linkage decreased with increasing α-CD in the preparation. This can be attributed to the inclusion complex of α-CD/coniferyl alcohol in the polymerized step. The prolonged addition of monomer with the addition of HRP every 24 h led to a further decrease in the 5-5′ linkage (entry 9). The relatively high yield of the 5-5′ structure with the addition of HRP at the start (entry 10) can be attributed to reduced regulation of the DHP structure by α-CD, which supported the results from ^{1}H NMR and thioacidolysis.

Scheme 1. Thioacidolysis and Raney Nickel Desulfurization of DHP



Scheme 2. Ozonation of the 8-*O*-4′ Linkage Formed via a Quinone Methide Intermediate

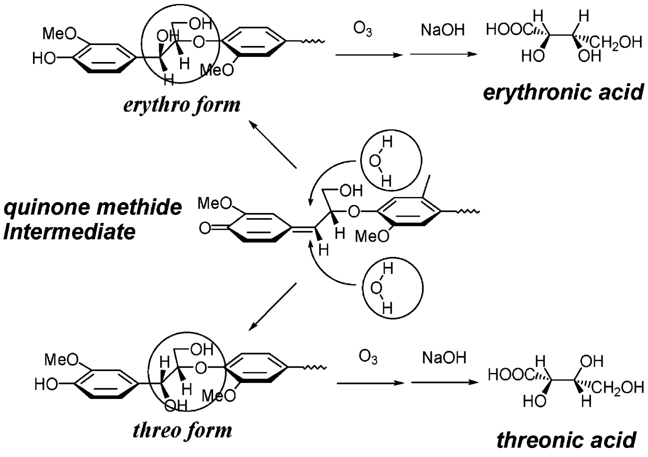
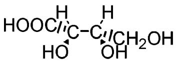
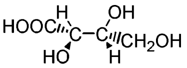


Table 3. Ratio of Erythronic Acid:Threonic Acid Determined by the Ozonation of DHP

		 $HOOC-C(H)(HO)-C(H)(OH)-CH_2OH$ erythronic acid	 $HOOC-C(H)(HO)-C(OH)(H)-CH_2OH$ threonic acid
entry in Table 1	template	*erythro*:*threo*	
6	none	52:48	
8	α-CD (1.5 equiv)	51:49	

Selective degradation of the aromatic nuclei of DHP by ozone gives low-molecular-weight compounds that retain the chiral structures of the original chiral side-chain structures of DHP. The quinone methide intermediate resulting from the 8-*O*-4′ linkage is nucleophilically attacked by the hydroxyl groups at the C_7 position (Scheme 2). Ozonation gives important information on the *erythro*/*threo* ratio of 8-*O*-4′ structures. For the determination of *erthro* and *threo* forms of 8-*O*-4′ structures, DHP prepared by method B1 was treated with ozone in acetic acid:H_2O:methanol (16:3:1 *v*/*v*/*v*) at 0 °C. The results are shown in Table 3. DHP prepared by the conventional end-wise method has a racemic side chain in its 8-*O*-4′ structures from the ozonation products. Addition of α-CD produced no change in the chiral side chain, which indicated that DHP prepared in α-CD has a racemic form. Addition of α-CD did not lead to

CDV

stereo-controlled formation of the 8-*O*-4′ linkage structure in DHP. The addition of compounds with chirality often gives stereo-controlled products. In lignin biosynthesis, woody polysaccharide templates do not regulate the stereo structure of side chains in the formation of the 8-*O*-4′ linkage. In the polymerization of coniferyl alcohol, the addition of carbohydrate polymers (oligomers) could not perhaps be expected to produce a stereo regulated structure in the polymerizate.

Roles of CDs in the Polymerization Step. In the present work, we used CDs for the enzymatic polymerization of coniferyl alcohol as additives. When CDs form the inclusion complex with coniferyl alcohol, a monolignol can be given the steric effect,[20,21] the polarity effect,[17,29,30] and the solubility effect[15,16,18] for DHP synthesis. The DHP synthesized in the α-CD system had an 8-*O*-4′-rich structure, which is due to the steric effect and the polarity effect. As mentioned above, the inclusion complex with α-CD inhibits coniferyl alcohol from the coupling reaction at the 5 position which has the ability of the formation of 8-5′ and 5-5′ linkages. In the α-CD system, the steric effect is an important factor of controlled structure of DHP. The 8-*O*-4′-rich structure with the decrease of 8-5′ and 5-5′ is a result from the steric effect. The increase of the 8-*O*-4′ linkage with the decrease of 8-5′ and 5-5′ linkages may also be attributed to another factor, i.e., the polarity effect. Since the cavity of α-CD is hydrophobic, it can change the electronic delocalization of the radical. Previous works have proposed that the hydrophobic environment gave the change of the electronic delocalization, which resulted in DHP having an 8-*O*-4′ rich structure.[28.29] On the other hand, the solubility effect was not so much important for the synthesis of DHP in the α-CD system. α-CD increases the solubility of coniferyl alcohol and DHP oligomers and thus may change the kinetic parameters of the polymerization. Actually, small amounts of α-CD (0.5 equiv for coniferyl alcohol) allowed the DHP with higher molecular mass than that prepared by the conventional method. However, addition of a greater quantity of α-CD (1.5 equivalents for coniferyl alcohol) did not increase the molecular weight of DHP because the polymerization was inhibited from the steric hindrance arising from the inclusion complex. The improvement of DHP solubility by α-CD addition did not necessarily lead to a higher molecular weight of DHP.

Conclusion

In enzymatic polymerization of coniferyl alcohol by using HRP/H_2O_2, the presence of α-CD in the polymerization medium led to DHP with 8-*O*-4′-richer linkages compared to that prepared using no additive method. The 8-*O*-4′ content was controlled by the quantity of α-CD to some extent. This observation was due to the inclusion complex of α-CD with coniferyl alcohol. Higher yields of thioacidolysis products supported the conclusion that α-CD regulated the chemical structure of DHP. DHP obtained in the α-CD system has the racemic form of 8-*O*-4′ structure, indicated by the ozonation products. These results present many implications in both applied and basic research field related to lignin polymerization process. For the development of environmental friendly materials, much attention is paid to resources derived from plants and wood. Lignin would play an important role as a resource from photosynthesis because of its abundant production. It is expected that the indication of the regulation of the chemical structure of lignin may lead to an understanding of the relationship between the structure and properties of lignin. Evaluation of the properties of DHP prepared here is currently in progress.

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