Dehydrogenative Co-polymerization of *d*-Catechin and Coniferyl Alcohol*

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Abstract——To elucidate the possible occurrence of co-polymers of phenolic extractives and lignin in woods *d*-catechin was dehydrogenated in the presence of coniferyl alcohol with peroxidase and H_2O_2. Molecular weight distribution, UV spectra in the presence and absence of $AlCl_3$, methoxyl content and the degradation of the DHP by acidolysis, and by $KMnO_4$ oxidation after methylation showed that the DHP is a co-polymer which may be linked at C5′ of B ripg of *d*-catechin and C5 of coniferyl alcohol.

Introduction

Lignification is generally known to occur in all woody cell walls including ray parenchyma cells, whereas biosynthesis of heartwood extractives which are present in the cell wall, mainly in the lumen of parenchyma and vessels in heartwood, seems to be limited to the ray parenchyma cells, especially to those in transition wood. However, it is known that in some trees cell walls of radial ray parenchyma in sapwood are not lignified and their lignification simultaneously occurs in transition wood during heartwood formation[1]. In such cases if phenolic extractives synthesized by parenchyma cells are translocated to the lignification site of cell walls, the co-polymerization of the extractives with lignin precursors via radical couplings mediated by peroxidase and H_2O_2 may possibly occur.

In the present paper co-polymerization of *d*-catechin, a typical phenolic extractive, with coniferyl alcohol was studied to elucidate the possible occurrence of co-polymers of phenolic extractives and lignin in cell walls.

Experimental

Preparation of dehydrogenation co-polymer of coniferyl alcohol and d-catechin——Coniferyl alcohol (199 mg) synthesized by the Freudenberg method[2] and a commercial *d*-catechin (183 mg) were dissolved in a little amount of acetone, and the solution was

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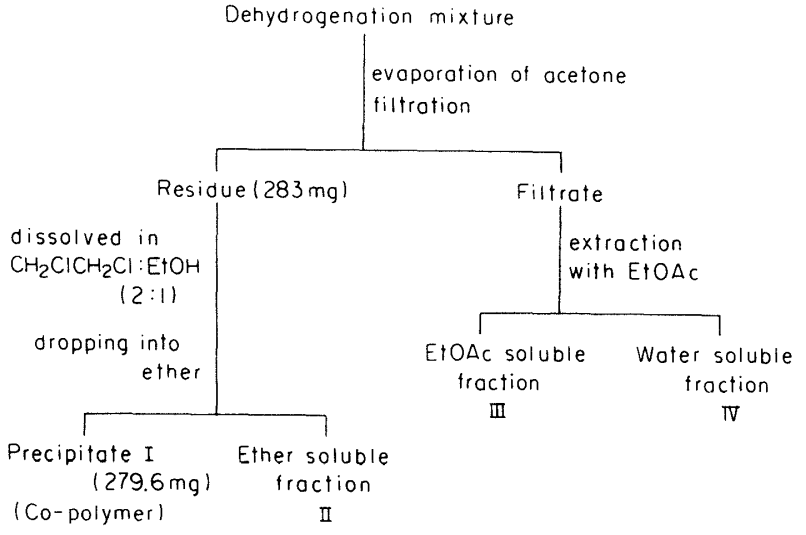

Fig. 1. Preparation scheme of dehydrogenation co-polymer of coniferyl alcohol and *d*-catechin.

diluted with 40 ml of 0.15 M phosphate buffer (pH 6.0). The solution and 0.1% H_2O_2 solution (68 ml) were dropped simultaneously into 10 ml of the phosphate buffer solution containing horse radish peroxidase (3 mg) for 24 hrs with stirring under nitrogen at room temperature. The reaction mixture was stirred for further 4~5 hrs, and the products formed were isolated and purified as shown in Fig. 1.

After evaporation of the acetone the precipitate (283 mg) was filtered and dissolved in 5 ml dichloroethane-ethanol (2:1). The solution was added dropwise into ether (100 ml) with stirring and the stirring was continued for 1 hr. The precitate (I) (280 mg) was obtained by filtration as a brown powder (yield, 73%), and the water-soluble products in the filtrate were separated into ethyl acetate soluble-(III), and the remaining water-soluble fractions (IV) respectively.

Methoxyl content of the precipitate (co-polymer) was determined by a micro-Zeisel method[3].

Molecular weight distribution——Molecular weight distribution of the co-polymer was estimated by high pressure liquid chromatography. The calibration curve to estimate the molecular weight was made using standard polystyrenes (MW 160000, 10000 and 4000), syrigaresinol and benzene as samples.

The mixture of the polystyrenes, syringaresinol and benzene dissolved in THF was applied to the high pressure liquid chromatography (Shimadsu-Deupone 830) at the following condition. Column, microstyragel 500 Å; carrier, THF; pressure, 40 kg/cm^2; UV detector. THF solutions of the co-polymer, the ether soluble fraction and the ethyl acetate soluble fraction were analyzed at the same condition.

UV spectra of the co-polymer, DHP and d-catechin——The effect of $AlCl_3$ on the spectra of the co-polymer, DHP of coniferyl alcohol and *d*-catechin was measured

immediately after the addition of six drops of the $AlCl_3$ solution which was prepared by dissolving 5 g of fresh anhydrous $AlCl_3$ to methanol (100 ml), to 2~3 ml of methanol solution of each sample in a cuvette. The $AlCl_3$/HCl spectrum was also recorded by addition of 3 drops of 12% HCl to the cuvette containing the sample with $AlCl_3$.

Acidolysis——Each 21 mg of the co-polymer and *d*-catechin was subjected to acidolysis in 2 ml dioxane-water (9: 1) containing 0.2 N HCl at reflux temperature for 4 hrs. The acidolysis monomers obtained were converted to their TMS derivatives and analyzed by gas-liquid chromatography. Column, 3% OV-17 on Chromosorb W 2 m; temperature, 195°C; FID detector.

Acidolysis products of the co-polymer were identified by comparing their retention times with those of authentic compounds and co-chromatography. The compounds corresponding to the respective peaks on the chromatogram were further analyzed by GC-MS (Shimadzu-LKB 9000)[4].

$KMnO_4$ oxidation——The co-polymer was ethylated with diethyl sulfate and NaOH, and the ethylated product was subjected to $KMnO_4$ oxidation. The oxidation products were extracted with acetone-chloroform and methylated with diazomethane. The methylated products thus obtained were analyzed by gas-liquid chromatography. Column, 3% SE-54 on Chromosorb W 2 m; temperature, 185°C; FID detector. The separated compounds were identified by comparing their retention times with those of authentic compounds and co-chromatography, and also by GC-MS[5].

Authentic 3, 4-diethoxymethyl benzoate was prepared by ethylation of protocatechuic acid to 3, 4-diethoxybenzoic acid and followed by methylation of the latter compound with diazomethane. 3-Methoxy-4-ethoxymethyl benzoate was also prepared by ethylation of vanillic acid with ethyl iodide and subsequent methylation of 4-ethoxyvanillic acid with diazomethane.

Results and Discussion

On the dehydrogenative co-polymerization of coniferyl alcohol and *d*-catechin the reaction mixture gave an orange color immediately after the dropping of H_2O_2 solution. The dehydrogenation co-polymer purified showed a pale brown color, and the yield was 73% indicating that the polymer is composed of both coniferyl alcohol and *d*-catechin.

Fig. 2 shows the calibration curve of molecular weight distribution by standard polystyrenes, syringaresinol and benzene. On the microstyragel 500 Å column, polystyrenes (MW 10000 and 4000) and syringaresinol showed a linearity between the molecular weight and the elution volume, but polystyrene (MW 160000) and benzene were out of the calibration curve. In the present investigation the micro-

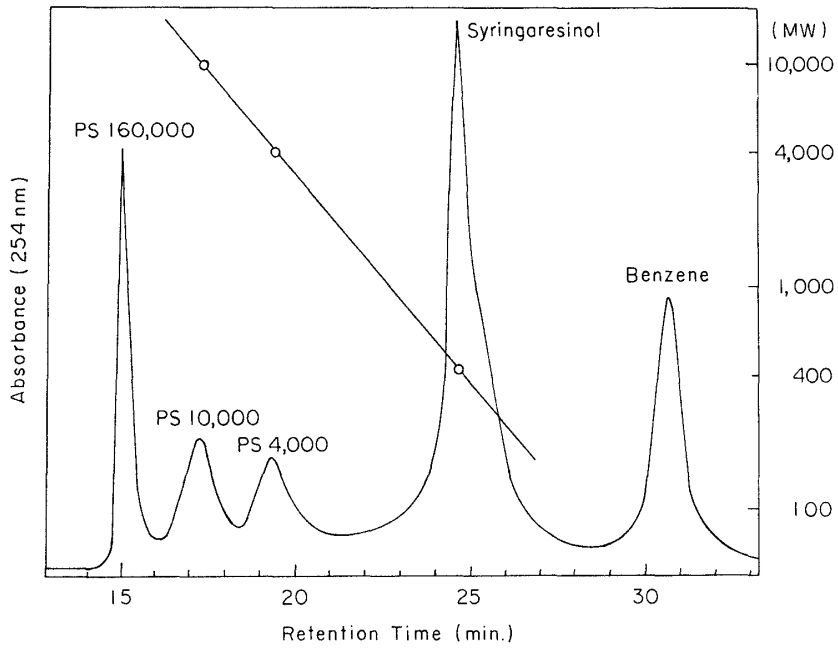

Fig. 2. Calibration curve for molecular weights of polystyrenes and syringaresinol.

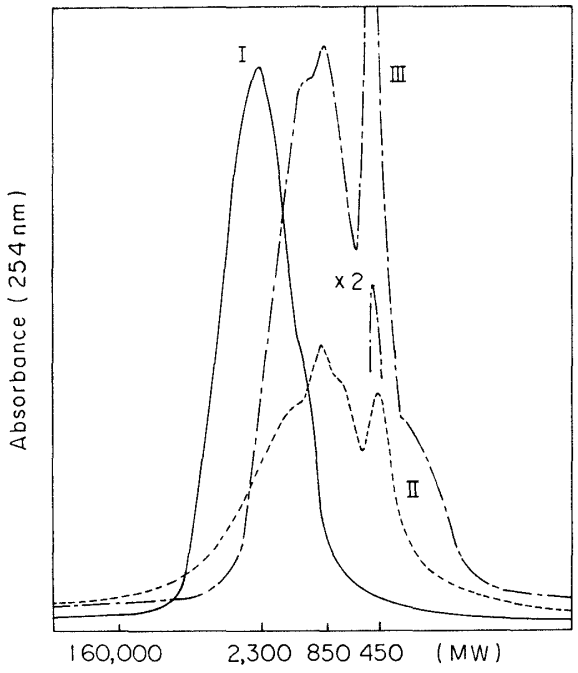

Fig. 3. Molecular weight distribution of I ····· Precipitate, II – – – – Et_2O soluble and III — — — EtOAc soluble fractions in dehydrogenative polymerization of coniferyl alcohol and *d*-catechin.

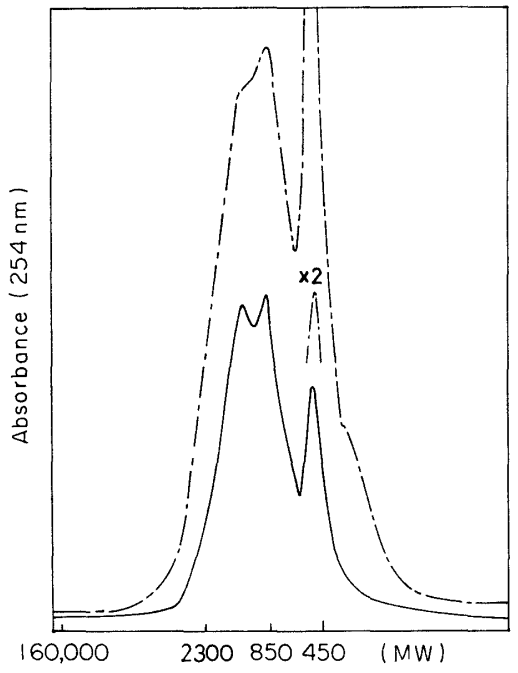

Fig. 4. Molecular weight distribution of EtOAc soluble fractions of the reaction mixture during dehydrogenation.
····· after 12 hrs
— — — after 30 hrs

styragel 500 Å column was tentatively used for the determination of molecular weight distribution of DHPs, although it is known that the molecular structure of lignin is not linear as in polystyrenes and therefore exact molecular weight can not be estimated.

Fig. 3 shows the molecular weight distribution of the co-polymer, the ether soluble-, and the ethyl acetate soluble fractions in dehydrogenative co-polymerization of coniferyl alcohol and *d*-catechin. The molecular weight of the co-polymer was about 2300, and in the most case the co-polymer gave rather a narrow peak of mono-dispersed system. While the ether soluble-, and the ethyl acetate soluble fractions gave two peaks at MW 850 and 450.

Fig. 4 shows the molecular weight distribution of the ethyl acetate soluble fractions of the reaction mixture in the middle and the end of dehydrogenation. It is evident that the ratio of the peak area of MW 450 fraction to that of MW 850 fraction increases during dehydrogenation, which suggests that MW 850 fraction polymerizes

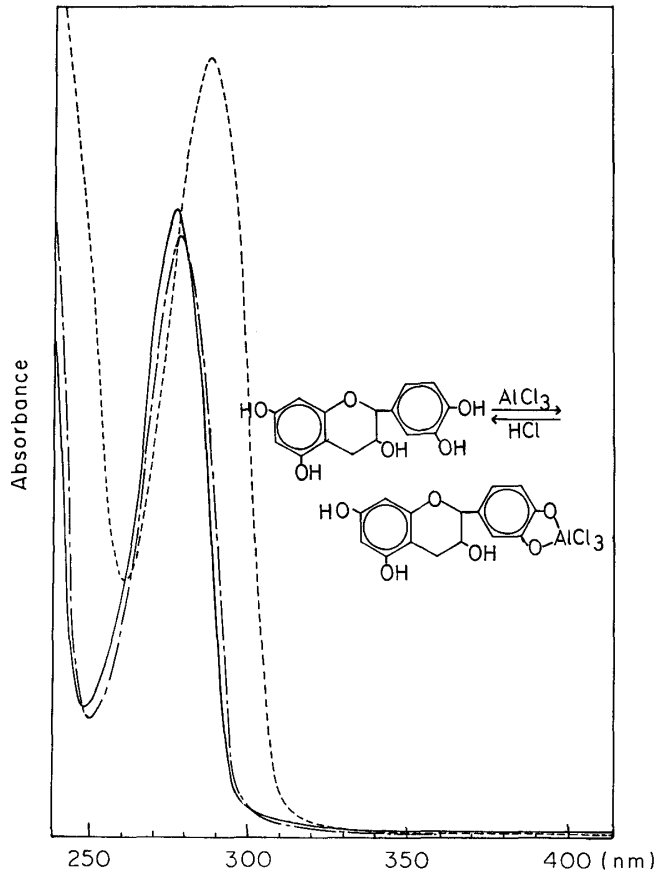

Fig. 5. Effect of $AlCl_3$ on the spectrum of *d*-catechin.
——— MeOH
- - - - MeOH+$AlCl_3$
— - — MeOH+$AlCl_3$+HCl

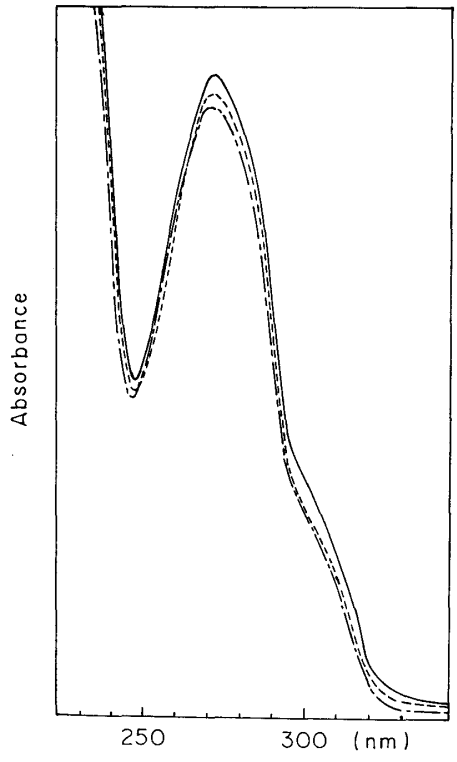

Fig. 6. Effect of $AlCl_3$ on the spectrum of coniferyl alcohol DHP.
——— MeOH
- - - - MeOH+$AlCl_3$
— - — MeOH+$AlCl_3$+HCl

faster to the more higher molecular weight fraction than the rate of polymerization of the fraction with MW 450.

$AlCl_3$ generally forms acid labile complexes with flavonoids which have *o*-dihydroxy systems; in addition of $AlCl_3$, flavones and flavonols which have hydroxyl groups at C3 or C5 form acid stable complexes. Therefore the presence of *o*-dihydroxyl group in the B ring of flavones and flavonoid can be detected to compare the spectrum of the flavonoid in the presence of $AlCl_3$ with that in $AlCl_3$/HCl[6].

Fig. 5 shows the effect of $AlCl_3$ on the spectrum of *d*-catechin. *d*-Catechin gives a peak at 280 nm in methanol due to both A and B rings. $AlCl_3$ shifts this peak to the longer wave length about 20 nm and in the addition of aqueous HCl the shifted peak returns to the former position.

Fig. 6 shows the effect of $AlCl_3$ on the spectrum of DHP of coniferyl alcohol. The spectrum is not changed in the addition of $AlCl_3$ because guaiacylglycerol-β-coniferyl ether, phenylcoumarane and pinoresinol structures in the DHP have no *o*-dihydroxyl group.

Fig. 7 shows the effect of $AlCl_3$ on the spectrum of dehydrogenation co-polymer. The spectrum of the dehydrogenation co-polymer in methanol has two peaks at 280 nm and 380 nm, and the latter absorption was shifted to the longer wave length about 30~40 nm by the addition of $AlCl_3$. Takino[7] reported that the mixture of epigallocatechin and epicatechin was oxidized to the colored substance with polyphenol

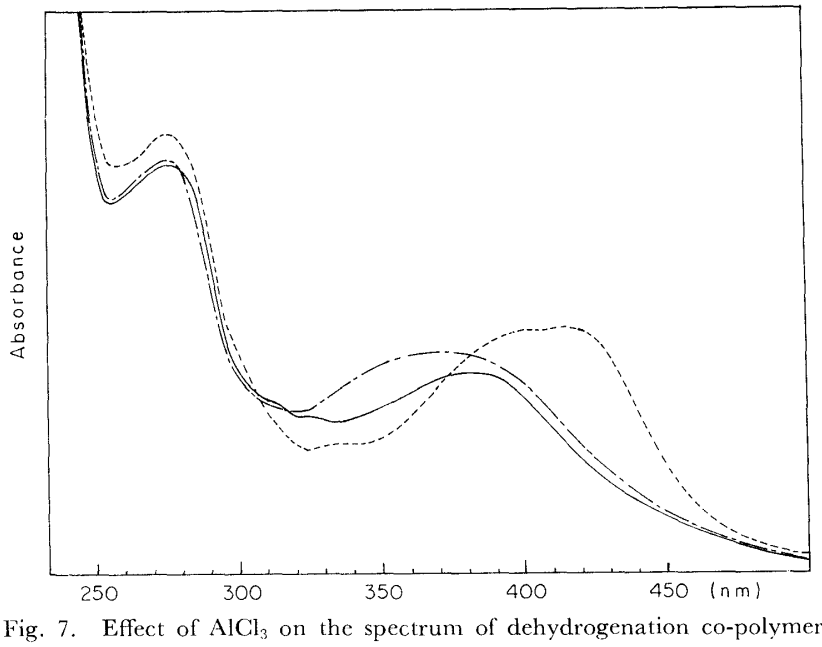

Fig. 7. Effect of $AlCl_3$ on the spectrum of dehydrogenation co-polymer of coniferyl alcohol and *d*-catechin.
MeOH
- - - - MeOH + $AlCl_3$
— — — MeOH + $AlCl_3$ + HCl

oxidase of tea leaves and potassium fericyanide-$NaHCO_3$. He isolated a pigment which gave strong absorptions near 280 nm and 370 nm, and suggested the pigment to be a benzotropolone compound.

However, in dehydrogenative co-polymerization of *d*-catechin and coniferyl alcohol, the formation of the tropolone nucleus is unlikely, and it is more reasonable to suppose that the absorption at 380 nm is due to the biphenyl structure and a conjugated double bond. Since the addition of $AlCl_3$ shifted the only absorption at 380 nm to the longer wave length, it is conceivably to suppose that the biphenyl structure was formed in the B ring of *d*-catechin. It was found that *d*-catechin alone was not polymerized with horse radish peroxidase-H_2O_2.

Fig. 8 shows ΔA spectrum of *d*-catechin and the dehydrogenation co-polymer between neutral and alkaline conditions. It is shown that both the co-polymer and *d*-catechin give absorptions at 430 nm which may be due to *o*-quinone, and at 300 nm. $\Delta\varepsilon$ of *d*-catechin and of the co-polymer at 430 nm were estimated to be 4590 and 11880 based on their molecular weights. These results evidently show that the co-polymer is composed of *d*-catechin and coniferyl alcohol.

The methoxyl group of the dehydrogenation co-polymer was found to be 8.3% which is less than that of the DHP of coniferyl alcohol (15.1%), and the ratio of coniferyl alcohol to *d*-catechin in the co-polymer is estimated to be 1.8 based on the methoxyl contents. From these results the average numbers of coniferyl alcohol

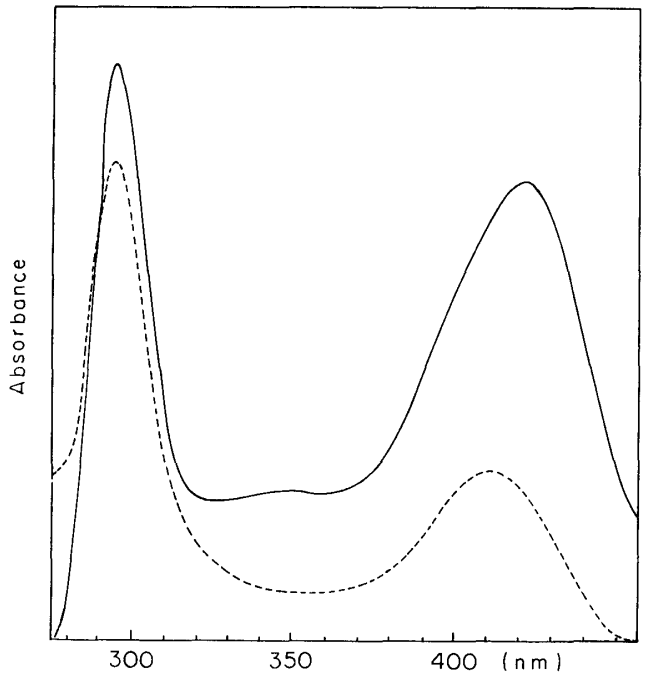

Fig. 8. ΔA-spectra of *d*-catechin, and co-polymer of coniferyl alcohol and *d*-Catechin.
——— *d*-Catechin - - - - Co-polymer

Table 1. The ratio of coniferyl alcohol and *d*-catechin components in the co-polymer.

	M W	OCH_3 (%)	$\Delta\varepsilon$ 430nm
Coniferyl alcohol DHP	(196/unit)	15.1	0
d-Catechin	290	0	4590
Co-polymer	2300	8.3	11880
Molar ratio of coniferyl alcohol and *d*-catechin in co-polymer		1.8	
Average number of coniferyl alcohol in co-polymer		6.4	
Average number of *d*-catechin in co-polymer		3.6	
d-Catechin with free *o*-diphenol in co-polymer			2.6
d-Catechin with etherified B-ring in co-polymer			1.0

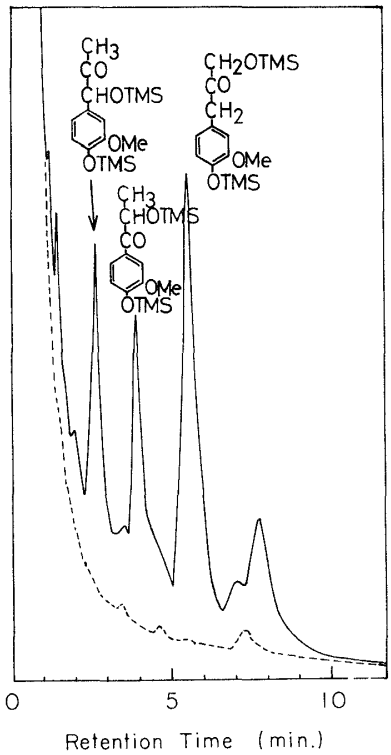

Fig. 9. Gas-liquid chromatogram of TMS derivatives of acidolysis monomers from co-polymer of coniferyl alcohol and *d*-catechin.
Co-polymer
d-Catechin

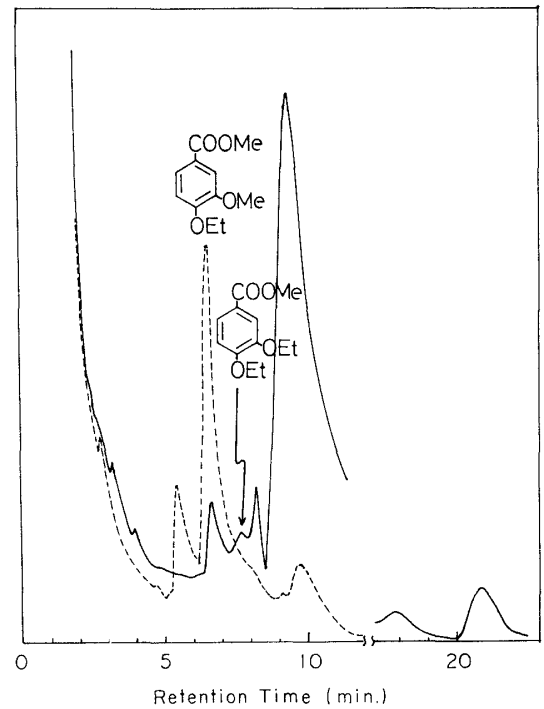

Fig. 10. Gas-liquid chromatogram of methylated $KMnO_4$-oxidation products of ethylated co-polymer of coniferyl alcohol and *d*-catechin and DHP of coniferyl alcohol.
Co-polymer
Coniferyl alcohol DHP

and *d*-catechin molecules constituting one molecule of the co-polymer were calculated (Table 1).

Fig. 9 shows the gas-liquid chromatogram of the TMS derivatives of acidolysis monomers of the co-polymer and *d*-catechin itself. The yields of the chloroform soluble fractions of acidolysis mixture of the co-polymer and of *d*-catechin were 56.2 and 24.5%, respectively. On the acidolysis of the co-polymer, 1-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-propanone, 2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone and β-oxyconiferyl alcohol were identified by GC-MS analysis, but any acidolysis monomer from *d*-catechin component was not detected. Therefore, no information on the linkages between coniferyl alcohol and *d*-catechin was obtained from the acidolysis experiment.

Fig. 10 shows the gas-liquid chromatogram of $KMnO_4$ oxidation products of the ethylated co-polymer and DHP of coniferyl alcohol. 3-Methoxy-4-ethoxymethyl benzoate, and 3, 4-diethoxymethyl benzoate which is evidently derived from the B ring of *d*-catechin component were identified.

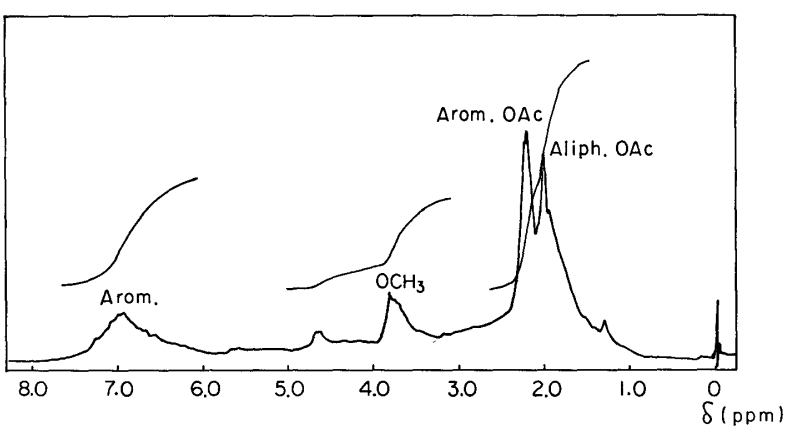

Fig. 11. NMR spectrum of acetylated co-polymer of coniferyl alcohol and *d*-catechin.

Fig. 11 shows the NMR spectrum of the acetylated co-polymer. The ratio of the amount of aromatic protons of the co-polymer to that of methoxyl protons is higher than that of the DHP of coniferyl alcohol, whereas the aliphatic alcohol groups of the co-polymer is less than phenolic ones indicating that a large amount of *d*-catechin is incorporated into the co-polymer. Pinoresinol, dehydrodiconiferyl alcohol and guaiacylglycerol-β-coniferyl ether were isolated and identified from the dimer fraction of both co-polymer and DHP of coniferyl alcohol by thin layer chromatography and NMR.

Weinges[8] isolated a crystalline dimer with the molecular formula $C_{30}H_{24}O_{12}$ and 8-hydroxy-(+)-catechin on the dehydrogenation of (+)-catechin with horse radish peroxidase-H_2O_2. However, these compounds could not be detected by thin

layer- and paper chromatography of the ethyl acetate soluble fraction of dehydrogenation of coniferyl alcohol and *d*-catechin in the present investigation.

As *d*-catechin scarcely gave a polymer by dehydrogenation under this condition, it is evident that *d*-catechin and coniferyl alcohol in the co-polymer are chemically bondend by C-C or C-O linkages, presumably at C5′ of B ring of *d*-catechin and C5 of coniferyl alcohol judging from UV and ^{13}C-NMR.

The fact that coniferyl alcohol and *d*-catechin gave a co-polymer with the same ratio of both components in the original reaction mixture strongly suggests that the lignin and phenolic extractives would give co-polymers, when lignification occurred in the ray parenchyma cells in transition wood where biosynthesis of phenolic extractives occurs simultaneously.

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