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Effect of Polysaccharides on Dehydropolymerization of Coniferyl Alcohol

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Abstract——Molecular weights of DHPs of coniferyl alcohol prepared in the presence of beech hemicellulose and pectic acid a little increased as compared with that of DHP prepared in the absence of the polysaccharides. However, the molecular weights of the DHPs were still about a half of that of conifer MWL. The amounts of degradation products and functional groups of the DHPs suggested a general resemblance in the mode of interphenylpropane linkages between the DHPs and conifer MWL. As main differences the DHPs gave higher amounts of coniferyl aldehyde and vanillin in acidolysis and of phenolic hydroxyl- and *p*-hydroxybenzyl alcohol groups.

Introduction

It is well known that coniferyl alcohol is converted into a dehydrogenation polymer (DHP) by mushroom laccase or plant peroxidases and that the properties of the DHP closely resemble spruce milled wood lignin (MWL) in almost all chemical aspects. However, molecular weight of the DHP has not been conclusively defined.

In earlier investigations FREUDENBERG et al.[1,2] found that the molecular weights of DHPs of coniferyl alcohol and of *p*-coumaryl alcohol determined by isothermic distillation were 810-820 and 670, respectively, and further they obtained evidence that the DHP of ferulic acid was composed of six molecules of ferulic acid.

On the other hand, NOZU[3] found that the molecular weight (Mn) of DHP obtained from coniferyl alcohol by a bamboo shoot peroxidase was approximately 1000, and that the attempts to increase the molecular weight of the DHP were unsuccessful.

Microscopic observations[4] have revealed that lignification is initiated in the primary wall adjacent to the corner of cells and that the hemicelluloses in the cell wall may play an essential role to make growing lignin[5]. On the other hand, FREUDENBERG[6] proposed a possible formation of lignin-carbohydrate linkages via quinone methide intermediates in enzymic dehydrogenation of coniferyl alcohol.

In the present investigation, coniferyl alcohol was dehydrogenated in the presence of various polysaccharides by horse radish peroxidase to examine both

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FREUDENBERG's hypothesis and a possible role of a DHP-polysaccharide complex to increase the molecular weight of the DHP.

Experimental

Coniferyl alcohol was synthesized by lithium aluminum hydride reduction of ethylferulate according to the FREUDENBERG's method[7]. β-Oxyconiferyl alcohol was prepared from acetylhomovanillic acid according to the FISCHER and HIBBERT's method[8]. M. p. 81-82°C (Lit. 82°C), Anal. Calcd. for $C_{10}H_{12}O_4$: C, 61.20 %, H, 6.17 %, Found : C, 61.08 %, H, 6.17 %. Molecular weight : 196 by Mass spectrometry. Hemicelluloses were prepared by extraction of holocellulose of Japanese cypress (*Chamaecyparis obtusa* ENDL.) and beech (*Fagus crenata* BLUME) wood powder with 4 % NaOH solution and by precipitation into a large volume of ethanol, respectively. A part of the hemicelluloses was hydrolyzed with 72 % sulfuric acid, and the monosaccharides formed were converted to their alditol acetates and analyzed by gas-chromatography according to the method of BORCHARDT et al[9]. The ratios of monosaccharides in both hemicelluloses were as follows: Japanese cypress, xylose, 51.0 %, mannose, 26.0 %, galactose, 8.0 %, arabinose, 6.0 %, and glucose, 7.0 %. Beech, xylose, 90.5 %, mannose, 2.5 %, arabinose, 2.0 %, glucose, 0.4 %, galactose, trace.

Horse radish peroxidase and other chemicals are commercial products.

Preparation of DHPs——In first experiments, coniferyl alcohol (0.5 mM), each of respective polysaccharides (30-150 mg) were added in 100 ml of a phosphate buffer solution (0.01 M, pH 6.0). Then horse radish peroxidase (1.5 mg) and 0.1 % solution of hydrogen peroxide (0.5 mM) were added into the solution successively, and the solution was kept at about 25°C under stirring for 24 hrs. Yellowish white precipitates formed were centrifuged (1500 rpm, 20 min.) and dried over P_2O_5 for 16 hrs. The dried DHP was dissolved in acetone containing 10 % water, the solution was centrifuged and the soluble portion was added in dropwise into benzene under stirring. The precipitated DHP was collected by centrifugation, dried completely over P_2O_5 and weighed. The acetone-insoluble fraction containing added polysaccharide was dissolved in dimethyl sulfoxide and the soluble portion was precipitated into ethanol.

In second experiments aqueous solution of coniferyl alcohol (10 mM, 800 ml) and 0.1 % solution of hydrogen peroxide (10 mM, 340 ml) in respective separating funnels were added simultaneously in dropwise under stirring into 300 ml of a phosphate buffer (0.05 M, pH 6.0) containing peroxidase (5 mg) and each of the polysaccharides (1.8 g). The reaction mixture was kept at about 25°C under stir-

ring, and after 24 hrs peroxidase (5 mg) and 0.1 % solution of hydrogen peroxide (5 mM) were added in a similar way, and the reaction was continued for 4 days.

In separate experiments the enzymic reaction was carried out under nitrogen atmosphere to examine the effect of oxygen for polymerization of the radicals of coniferyl alcohol, and further a non-enzymic dehydrogenation reaction was carried out by using a redox system (Fe^{++}-H_2O_2). The DHPs thus formed were purified as described above.

Determination of Molecular Weight of DHPs——Suitable amounts (10-50 mg) of DHP were dissolved in methyl cellosolve, and the molecular weight (Mn) was determined by using a MECHROLAB vapor pressure osmometer. For calculation of the molecular weight of the DHP a calibration curve obtained by using curcumin as a standard compound was used[10].

Determination of Phenolic Hydroxyl Group——The amounts of phenolic hydroxyl groups of the DHP and MWL were determined spectrophotometrically by $\Delta\varepsilon$-method according to the procedure of GOLDSCHMID[11].

Determination of α-Carbonyl, p-Hydroxybenzyl Alcohol and p-Alcoxybenzyl Alcohol Groups——The DHPs and the MWL were methylated with diazomethane and the methylated samples were dissolved in dioxane and reduced with sodium borohydride. From $\Delta\varepsilon r$ value at 310 nm obtained from the difference spectrum between methylated and its reduced sample the amount of α-carbonyl group was calculated. The DHP and the MWL methylated with diazomethane were oxidized with DDB (2, 3-dichloro-5, 6-dicyan-1, 4-benzoquinone) in dioxane at room temperature for 3 days in the dark, and the oxidized samples were precipitated into ether. A part of the oxidized samples was dissolved in dioxane, diluted with ethanol, and reduced with sodium borohydride in 0.02 N NaOH at room temperature for 24 hrs. From the UV spectra of both oxidized and reduced methylated samples $\Delta\varepsilon r$ value at 310 nm was obtained and the amount of α-carbonyl group produced with DDB oxidation was calculated according to the procedure of ADLER et al[12].

p-Hydroxybenzyl Alcohol Group——The DHPs and the MWL were dissolved in methyl cellosolve and subjected to the reaction with quinonemonochlorimine at room temperature for 1 hr in the dark. The optical density of the reaction solution was determined at 630 nm and the amount of *p*-hydroxybenzyl alcohol group was calculated based on the calibration curve obtained with guaiacol[12,13].

Alkaline Nitrobenzene Oxidation——Each 50 mg of the DHPs or MWL, 2 ml of 2 N NaOH and 0.1 ml of nitrobenzene were added in a small stainless steel bomb (10 ml volume) and heated at 165°C for 2 hrs in an oil bath. Aromatic aldehydes produced were extracted with ether and analyzed by gas-chromatography. Column, 20 % DC 550 on chromosorb W, 1 m; temperature, 194°C; FID detecter. The

amount of vanillin was calculated based on the calibration curve prepared previously.

Acidolysis——Each 100 mg of the samples was subjected into acidolysis in dioxane-water (9 : 1) containing 0.2 N HCl at reflux temperature for 4 hrs[14]. The reaction mixture was added in dropwise into water under stirring and the precipitated substances were removed by centrifugation. The aqueous solution was extracted with ether and the ether was evaporated. The residue was dried over P_2O_5, converted to their trimethyl silyl derivatives as usual and analyzed by gas-chromatography. Column, 3 % SE-52 on chromosorb W, 2 m or 2 % OV-17 on chromosorb W, 2 m. Temperature, 195°C; and 180°C FID detecter. The amounts of β-oxyconiferyl alcohol and its derived products were calculated by using the calibration curves prepared for respective authentic compounds.

Results and Discussion

Molecular Weight——The yields of DHPs were generally 50-65 % of coniferyl alcohol, although DHP prepared in the presence of Japanese cypress hemicellulose in second experiment yielded 45-35 % exceptionally. SIEGEL[15] found that eugenol was dehydrogenated to a lignin-like polymer by a peroxidase adsorbed onto a filter paper, but not in the absence of cellulose, and he further found that partly acetylated cellulose, in which one-quarter of the cellulosic hydroxyl groups were blocked, lost more than half of the lignin-forming capacity. From these results SIEGEL concluded that hydrogen bonding of the precursor to the cell wall or precursor-polysaccharide interaction are essential factors in lignin formation, whereas dehydrogenation in homogeneous solution favors dimerization instead.

Table 1. Yield and molecular weight of DHPs.

	Polysaccharide added (mg)	DHP Yield (%)	Molecular weight (Mn)
Control	0	63	1024
Pectin	60	59	1024
	150	63	1036
Pectic acid	60	51	1189
	150	66	1271
Hemicellulose (Beech)	60	50	1127
	150	58	1194
Hemicellulose (Japanese cypress)	60	46	1032
	150	50	1075
Cellulose powder (Whatman)	60	62	1024
	150	63	1037
MWL (*Thuja Standishii*)			2580

However, the yield of DHP of coniferyl alcohol was not affected by the presence of polysaccharides, and as shown in Table 1 the molecular weight (Mn) of the DHP prepared in the absence of polysaccharide (control) was 1024 which corresponded to the value obtained by NOZU[3]. On the other hand, the molecular weights of the DHPs prepared in the presence of beech hemicellulose and pectic acid were a little higher. Then, these polysaccharides may contribute partly for increasing the molecular weights of the DHPs. The molecular weight of a conifer (*Thuja Standishii* CARR.) MWL which was used as a standard substance was 2580.

MARTON[10] obtained the molecular weight (Mn) of spruce MWL, 2200 by using the same type of vapor pressure osmometer and calculated the molecular weight (Mw) to be 4400 based on the ratio, Mw/Mn, 2. HIROI and MIYAZAKI[16] also determined the Mn of Japanese cypress MWL to be 4700 by a vapor pressure osmometer (HITACHI type 115) and calculated the ratio, Mw/Mn to be 3.72.

It appears that the ratio, Mw/Mn in conifer MWLs is generally between 2 and 4. Applying the average value of the ratio, 3 to the values of Mn of the DHP (control), DHP (pectic acid) and DHP (beech hemicellulose) the values of Mw are 3072 (16 mer), 3813 (21 mer) and 3582 (19 mer), respectively, which may indicate an increasing effect of these polysaccharides for dehydropolymerization of coniferyl alcohol.

On the other hand, the molecular weight (Mn) of the DHP prepared under nitrogen atmosphere and by catalysis of a non-enzymic redox system were 1080 and 1510*, respectively. These results seems to indicate that oxygen did not inhibit the radical polymerization of coniferyl alcohol, and that the non-enzymic redox system catalyzed the dehydropolymerization of coniferyl alcohol in a similar way as in enzymic reaction.

Although the molecular weights of DHPs prepared with a certain polysaccharide a little increased the values were still about a half of that of conifer MWL suggesting the difference of dehydropolymerization *in vitro* from lignification of cell wall. In lignification, coniferyl alcohol will be dehydrogenated by a peroxidase bound with cytoplasmic membrane, radicals or quinone methids may combine in early stages with hemicelluloses located adjacent to the cytoplasmic membrane and subsequently dehydrogenation of the coniferyl alcohol will continue in the colloidal solution to make growing lignin. A more skilful technique should be required to establish the role of the polysaccharides to make growing lignin.

A higher molecular weight was expected for the DHP which was soluble in dimethyl sulfoxide but the yield was too small and further investigation could not

* Determined by using an ebulliometer.

be carried out, unfortunately.

Phenolic Hydroxyl Group——Table 2 shows the amounts of phenolic hydroxyl group determined spectrophotometrically. The amounts of phenolic hydroxyl group of the DHPs were between 0.36 and 0.40/OCH_3 and variation in the amounts between DHPs was little. However, the amount was much higher than that of a conifer (*Thuja*) MWL, 0.28/OCH_3.

Table 2. Phenolic hydroxyl group of DHPs.

	Polysaccharide added (mg)	Phenolic OH/OCH_3
Control	0	0.40
Pectin	60	0.39
	90	0.39
Pectic acid	60	0.38
	90	0.36
Hemicellulose (Beech)	60	0.36
	90	0.36
Hemicellulose (Japanese cypress)	60	0.40
	90	0.38
Cellulose powder (Whatman)	60	0.38
	90	0 40
MWL (*Thuja Standishii*)		0.28

FREUDENBERG[17] found that the $\Delta\varepsilon$-curve of DHP of coniferyl alcohol was identical to that of spruce MWL and the amount of phenolic hydroxyl group of the DHP was 0.3/OCH_3. However, in the present experiment the amounts of phenolic hydroxyl group of the DHPs were much higher than that of the conifer MWL which corresponded to lower molecular weights of the DHPs comparing with that of conifer MWL.

α-Carbonyl-, p-Hydroxybenzyl Alcohol and p-Alcoxybenzyl Alcohol Groups——As shown in Fig. 1 the difference of UV spectra between methylated DHP (a) and the methylated DHP reduced with sodium borohydride (b) was little, and the absorption around 310-350 nm by the DHP ascribing mainly to α-carbonyl group was quite low. On the other hand, the spectrum of DHP oxidized with DDB (c) gave a considerably high optical density around 310-350 nm ascribed to the newly formed α-carbonyl groups, and the absorption decreased to the state of original spectrum by sodium borohydride reduction (d).

The amounts of these functional groups calculated from these spectral analyses are shown in Table 3. The amounts of α-carbonyl group of the DHPs were about 0.02/OCH_3 which did not vary between the DHPs, whereas the amount of α-carbonyl group of the conifer MWL was 0.05/OCH_3. The amounts of *p*-hydroxybenzyl alcohol

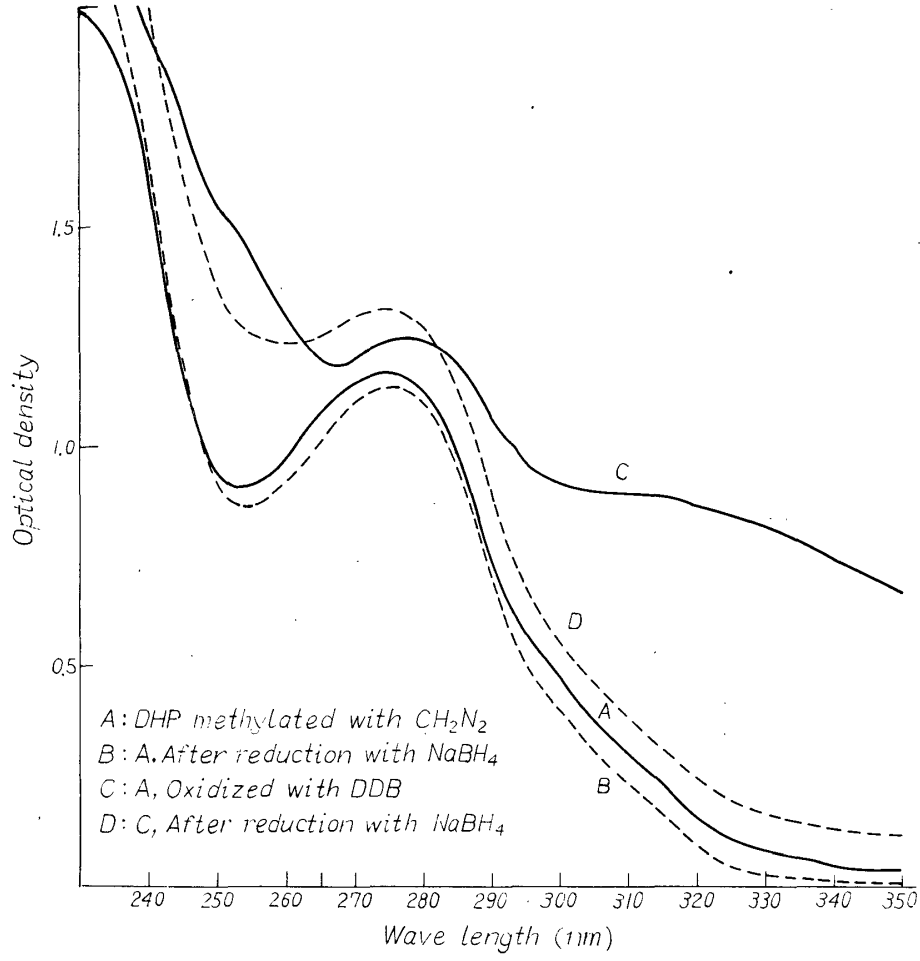

Fig. 1. UV spectra of DHP (control).

Table 3. α-Carbonyl, p-hydroxybenzyl alcohol and p-alcoxybenzyl alcohol groups in DHPs.

DHP*	α-Carbonyl group/OCH_3	p-Hydroxybenzyl alcohol/OCH_3	p-Alcoxybenzyl alcohol/OCH_3
Control	0.03	0.06	0.10
Pectin	0.02	0.06	0.11
Pectic acid	0.02	0.07	0.11
Hemicellulose (Beech)	0.02	0.07	0.11
Hemicellulose (Japanese cypress)	0.02	0.07	0.10
Cellulose powder (Whatman)	0.02	0.06	0.10
MWL (*Thuja Standishii*)	0.05	0.05	0.09

* DHPs prepared in the presence of 1.8 g of polysaccharides.

groups of the DHPs and the MWL were 0.06-0.07/OCH_3 and 0.05/OCH_3, respectively. And the amounts of p-alcoxybenzyl alcohol groups were 0.09–0.11/OCH_3 both in the

DHPs and the MWL, which corresponded to the amounts obtained for spruce MWL by ADLER et al[12].

Alkaline Nitrobenzene Oxidation——Table 4 shows the yields of vanillin from the DHPs. The yields were 23.0-25.0 % corresponding to the value, 23.6 % from *Thuja* MWL.

Table 4. Yield of vanillin from DHPs on nitrobenzene oxidation.

DHP*	Vanillin (%)
Control	25.0
Pectin	23.2
Pectic acid	23.0
Hemicellulose (Beech)	23.8
Hemicellulose (Japanese cypress)	23.2
Cellulose powder (Whatman)	25.0
MWL (*Thuja Standishii*)	23.6

* DHPs prepared in the presence of 1.8 g of polysaccharides.

Acidolysis——Fig. 2 shows typical gas-chromatograms of acidolysis monomers of the DHP (control) and *Pinus* MWL. β-Oxyconiferyl alcohol, 1-hydroxyl-1-(4-hydroxy-3-methoxyphenyl)-2-propanone, 2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone, guaiacylacetone, vanilloyl methyl ketone, coniferyl aldehyde, vanillin, vanillic acid, *p*-hydroxybenzoyl methyl ketone and *p*-hydroxybenzaldehyde were identified by co-chromatography with authentic compounds and their retention times using two different columns.

Table 5 shows the amounts of acidolysis monomers calculated from respective peak areas on the chromatograms by using calibration curves of the respective authentic compounds.

In the DHPs the amount of β-oxyconiferyl alcohol was highest followed by 2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone, coniferyl aldehyde, vanillin and 1-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-propanone successively. However, in the conifer MWLs the order of the amounts of acidolysis monomers were β-oxyconiferyl alcohol, 2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone and 1-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-propanone. The amounts of coniferyl aldehyde and vanillin were considerably lower than those from the DHPs.

KRATZL[17] found that coniferyl aldehyde was produced from guaiacylglycerol-β-aryl ether component in aqueous hydrolysis of spruce lignin and DHP of coniferyl alcohol and that the aldehyde was converted to vanillin.

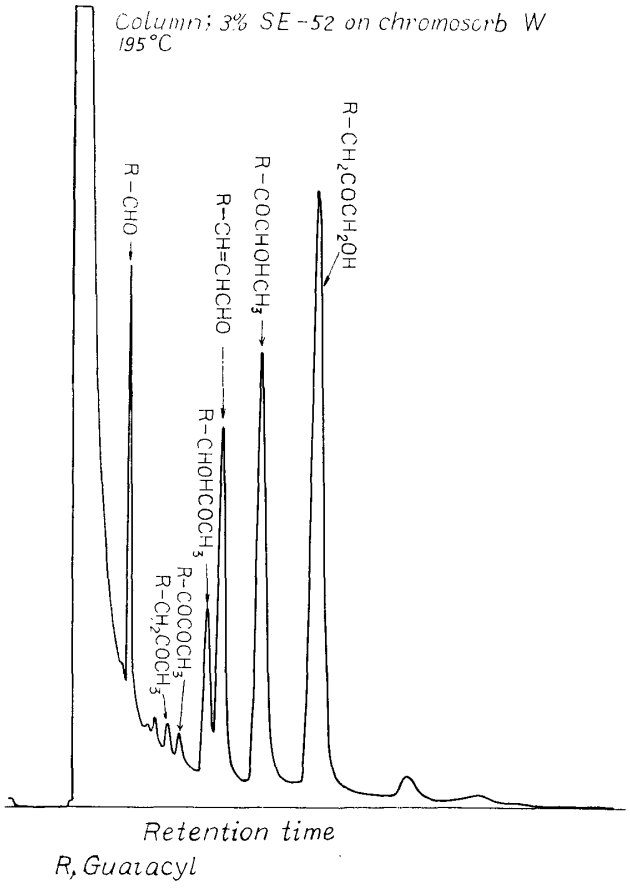

Fig. 2-a. Gas-chromatogram of trimethyl silyl derivatives of acidolysis monomers of DHP (control).

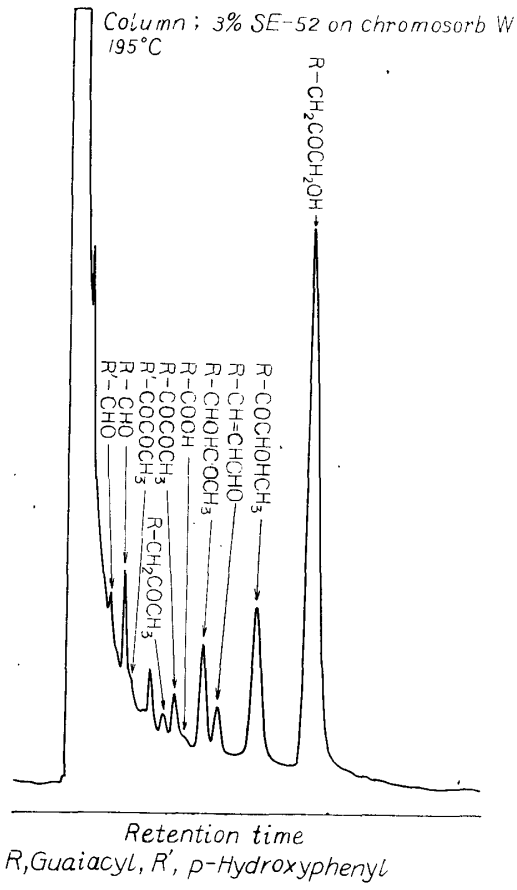

Fig. 2-b. Gas-chromatogram of trimethyl silyl derivatives of acidolysis monomers of *Pinus densiflora* MWL.

The lower amounts of acidolysis monomers, especially of coniferyl aldehyde and vanillin in the MWLs indicated a significant structural difference from the DHPs. One of the reason may be due to relatively lower amounts of guaiacylglycerol-β-aryl ether component involved, or a lower productivity of the component for the acidolysis products by their C-C linkages to neighboring phenylpropanes in more polymerized molecule of the MWL.

Conclusion

Molecular weights of DHPs of coniferyl alcohol prepared in the presence of beech hemicellulose and pectic acid a little increased as compared with that of DHP prepared in the absence of the polysaccharides. The yields of oxidation- and acidolysis products suggested a close resemblance in the mode of interphenylpropane likages between the DHPs and conifer MWLs. However, the amounts of coniferyl aldehyde and vanillin in acidolysis and of phenolic hydroxyl- and *p*-hydroxybenzyl

Table 5. Yield of acidolysis monomers from DHPs.

DHP*	R-** CH_2COCH_2OH	R- $COCHOHCH_3$	R- $CHOHCOCH_3$	R- CH_2COCH_3	R-$COCOCH_3$	R- $CH{=}CHCHO$	R-CHO	Total amount
	(Per cent of DHPs)							
Control	2.7	1.8	0.5	0.1	0.1	1.5	0.8	7.5
Pectin	2.5	1.8	0.7	0.1	0.1	1.4	0.7	7.3
Pectic acid	2.9	1.6	0.7	0.1	0.1	1.5	0.8	7.7
Hemicellulose (Beech)	2.7	1.6	0.6	0.1	0.1	1.3	0.8	7.2
Hemicellulose (Japanese cypress)	2.6	1.5	0.5	0.05	0.03	1.4	0.6	6.7
Cellulose powder (Whatman)	2.5	1.4	0.5	0.04	0.03	1.4	0.7	6.6
MWL (*Thuja Standishii*)	2.1	1.0	0.4	0.2	0.2	0.2	0.3	4.4
MWL (*Pinus densiflora*)	2.3	0.9	0.4	0.2	0.2	0.2	0.3	4.5

* DHPs prepared in the presence of 1.8 g of polysaccharides.

** R, Guaiacyl.

alcohol groups of the DHPs were considerably higher than those of the MWLs relating a less polymerization degree. These results indicated differences between DHP formation *in vitro* and lignification of cell wall in plants. Dimethyl sulfoxide-soluble fractions of the DHPs prepared in the presence of the polysaccharides gave a positive phloroglucinol reaction suggesting the presence of a DHP-polysaccharide complex, but the amount was too small and further investigation could not be carried out.

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