

# Dehydrogenative Polymerization of Monolignols by Peroxidase and H<sub>2</sub>O<sub>2</sub> in a Dialysis Tube. I. Preparation of Highly Polymerized DHPs\*

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**Abstract**—Coniferyl alcohol gave a highly polymerized DHP whose molecular weight was higher than that of bamboo MWL by dialysis membrane method. The DHPs obtained by this method were almost insoluble in any solvents but swelled in DMSO, DMF, formamide and pyridine. The molecular weight of soluble fraction of the DHPs showed to be higher than that of the DHP prepared by “Zutropf” method on gel filtration chromatography with Sephadex LH-60 but smaller than that of bamboo MWL with Sephadex G-200. However, thermal softening point (Ts) (175°C) of the total DHP obtained by dialysis membrane method was higher than that of bamboo MWL (160°C). Thermal softening points of DHPs were shifted to the higher temperature with increase of peroxidase concentration in the dialysis tube. The results seem to indicate that a concentrated enzyme solution is required to prepare the highly polymerized DHP.

## 1. Introduction

The dehydrogenative polymerization of lignin precursors (coniferyl, sinapyl and *p*-coumaryl alcohols) to lignin is a complex process, the reaction mechanism of which is almost impossible to be elucidated by *in vivo* experiment. Current knowledge of this polymerization processes is mainly due to the characterization of dehydrogenation polymers (DHP) of these cinnamyl alcohols carried out by Freudenberg *et al.*<sup>1-5)</sup> As a consequence of these studies, most of the known structural detail of lignins was elucidated based on the understanding of coupling modes of monolignols and of nucleophilic addition reactions to quinonemethides which were formed during dehydrogenative polymerization.

Freudenberg *et al.*<sup>6)</sup> found that the DHP of coniferyl alcohol closely resembles spruce lignin in almost all aspects in functional group contents, spectral characters and yields of various degradation products, and they called DHP artificial lignin. However, molecular weight of the DHP has not been conclusively defined. DHPs of coniferyl alcohol obtained by either “Zulauf” and “Zutropfverfahren” have been used effectively as model of lignin substructure and of low molecular weight

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lignin. However, these DHPs are inadequate as model for investigation on the highly polymeric characteristics of lignin, and for the biosynthetic process to give rise a gigantic lignin polymer grafted onto hemicelluloses.

Attempts have been made to increase the molecular weight of DHP; Higuchi *et al.* prepared DHP of coniferyl alcohol in the presence of various polysaccharides by horse radish peroxidase to examine a possible role of a DHP-polysaccharide complex to make increase the molecular weight of DHP.<sup>7)</sup> Molecular weights of DHPs 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 half of that of conifer MWL which was smaller than natural lignin.

Waymann *et al.* prepared DHP of coniferyl alcohol by an improved "Zutropf" method; substrates were added very slowly in peroxidase solution<sup>8)</sup>. Molecular weight of their DHP was found as high as spruce MWL. However, the DHPs obtained by their method were still much smaller than natural lignin and soluble in several organic solvents. Freudenberg<sup>6)</sup> and Harkin<sup>9)</sup> briefly discussed the difficulty of the formation of high polymer *in vitro* experiment and a possible formation of polyignols at the surface of the cell wall polysaccharide.<sup>10,11)</sup> Because the solubility of di-, tri- and tetralignols in aqueous solution is very low, these lignols precipitate out during dehydrogenation and then it is unlikely that the dehydrogenation of oligolignols is continued further.

However, Brown *et al.* reported that the enzyme liberated lignin was insoluble in most organic solvents and only that 25% of the lignin was dissolved in formamide<sup>12)</sup>. The molecular weight of natural lignin seems to be extremely higher than these DHPs and MWLs. Thus it is necessary to synthesize a highly polymerized and insoluble DHP to elucidate chemical and physical properties of lignin high polymer.

## 2. Experimental

### 2.1 *Dehydrogenative polymerization of coniferyl alcohol using a cellulose dialysis tube (Dialysis membrane method)*

#### 2.1.1 *Preparation of DHP by the dialysis membrane method*

Five mg of horse radish peroxidase (125–200 purpurogallin u/mg) was dissolved in 15 ml of 1/30 N phosphate buffer (pH 6.1), and the solution was packed into a cellulose dialysis tube (Type 36/32). The tube was then put into a flask which contained coniferyl alcohol solution in phosphate buffer (300 mg/1000 ml). Hydrogen peroxide (0.4 ml, 30% solution) was then added into the flask and the solution was stirred for 48 hr at room temperature (Fig. 1). Then a new tube which contained the same peroxidase solution was replaced and the reaction was continued for more 48 hr. The

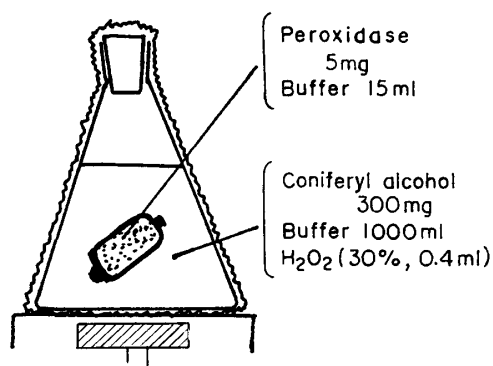


Fig. 1. Preparation method of DHP by dialysis membrane method

precipitates formed in the tube were washed with water to remove the low molecular weight materials and peroxidase. The amounts of the DHPs obtained in the first and the second tubes were 58 mg and 32 mg, respectively. Total yield of the twice reactions was 30%.

### 2.1.2 Effect of enzyme concentration on the yield of DHP

Each weight (0.05, 0.1, 0.2, 0.5, 1.0, 5.0, and 10.0 mg) of horse-radish peroxidase was dissolved in each of 10 ml phosphate buffer (1/30 N, pH 6.1) and packed into each cellulose dialysis tube (Type 20/32). These tubes were put into the same solution of coniferyl alcohol at once. The solution was stirred for 48 hr at room temperature and the reaction tubes were treated as described in 2.1.1. The yields of DHPs are shown in Table 1.

Table 1. Effect of enzyme concentration on the yields of DHP

Expt.	Reroxidase (mg)	Buffer (ml)	Ratio of enzyme concentration	Yield of DHP (mg)	Ratio of DHP yield
7	0.05	10	1	4.1	1.00
6	0.10	10	2	7.4	1.80
5	0.20	10	4	9.2	2.24
4	0.50	10	10	8.8	2.15
3	1.0	10	20	10.3	2.51
2	5.0	10	100	14.4	3.51
1	10.0	10	200	16.7	4.07

### 2.1.3 Effects of amounts of enzyme and buffer on the DHP yield

Each concentration and amount (5 mg/5 ml, 5 mg/10 ml, 2.5 mg/2.5 ml, and 2.5 mg/5 ml) of peroxidase in buffer solution was packed into each dialysis tube (Type 20/32) which was then subjected to DHP formation under the same condition as 2.1.2. The yields of DHPs were shown in Table 2.

## 2.2 Comparison of DHPs prepared by three methods (dialysis membrane method, "Zutropfverfahren" and "Zulaufverfahren")

Table 2. Yields of DHPs of coniferyl alcohol by the dialysis membrane method in the different conditions

Sample No.	Weight of Peroxidase	Volume of Buffer	DHP Yield
(1)	5.0mg	5.0ml	19.3mg
(2)	5.0	10.0	24.6
(3)	2.5	2.5	9.7
(4)	2.5	5.0	15.0

Weight of peroxidase=const. (2)/(1)=1.27

(4)/(3)=1.55

Volume of buffer = const. (1)/(3) 1.29

Concentration=const. (2)/(4) 1.64

(1)/(3) 1.99

### 2.2.1 Dialysis membrane method

Each 5 mg/10 ml of peroxidase/buffer solution was packed into a cellulose tube (Type 20/32) and the tube was put into a flask which contained 300 mg/10 ml/500 ml of coniferyl alcohol/acetone/phosphate buffer solution. Then 0.5 ml of H<sub>2</sub>O<sub>2</sub> solution (30%), was added to the flask. The tubes were kept for 0.5, 1, 3, 12, and 36 hr in the flask, respectively with stirring. The precipitates in tubes were then filtered, washed with water and lyophilized.

### 2.2.2 Zutropfverfahren

Three solutions (a, b and c) were prepared as follows. (a) 300 mg of coniferyl alcohol was dissolved in 10 ml of acetone and the solution was diluted with 100 ml of phosphate buffer. (b) 0.5 ml of H<sub>2</sub>O<sub>2</sub> solution (30%) was diluted with 100 ml of the buffer. (c) 5 mg of peroxidase was dissolved in 300 ml of the buffer. Then the solutions (a) and (b) were dropped into the solution (c) over a period of 20 hr and the reaction was continued more 16 hr. After the dropping was started, each 10 ml of the solution was taken out of the reaction mixture at the same reaction time as 2.2.1, respectively and the precipitates were treated according to 2.2.1.

### 2.2.3 Zulaufverfahren

The same solutions (a, b and c) as in 2.2.2 were mixed at once and the mixed solution was stirred for 36 hr. After the mixing was started, each 10 ml of the solution was taken out of the reaction mixture as in 2.2.2 and the precipitates were treated according to 2.2.1.

### 2.3 Preparation of DHP of sinapyl alcohol (*s*-DHP) and mixed DHP of coniferyl and sinapyl alcohols (*c·s*-DHP)

The dialysis tubes containing peroxidase (same as 2.1) were put into each buffer solution of 300 mg of sinapyl alcohol, and coniferyl and sinapyl alcohol (1:1) which were respectively dissolved in 10 ml of acetone and diluted with 300 ml of phosphate buffer. After 0.2 ml of 30% solution of H<sub>2</sub>O<sub>2</sub> was added, the solution was stirred for

48 hr at room temperature. The reaction mixtures were centrifuged and the precipitates were washed twice with water, and then DHPs obtained were suspended in dioxane-water (9:1) and lyophilized.

#### 2.4 Acidolysis of DHPs

Each 10 mg of DHPs was suspended in 10 ml of a mixture solution of dioxane and water (9:1) containing 0.2 N hydrogen chloride and refluxed for 4 hr under nitrogen. The reaction mixture was added dropwise into 100 ml of water with stirring and adjusted to pH 4 with 0.4 N sodium bicarbonate. Precipitates were centrifuged off and the acidolysis products in a supernatant solution were extracted with chloroform. The acidolysis products extracted were determined by gas chromatography according to the procedure of Higuchi *et al.*<sup>13)</sup> The gas chromatograms are shown in Fig. 9.

#### 2.5 Measurement of the properties of DHPs

##### 2.5.1 Molecular weight distributions

Molecular weight distribution of soluble fraction of DHPs were measured by Sephadex LH-60 with DMF and Sephadex G-200 with formamide.

##### 2.5.2 UV, <sup>1</sup>H-NMR, <sup>13</sup>C-NMR and IR spectra

UV and <sup>1</sup>H-NMR spectra of the DMF soluble fractions of the DHPs were measured by a HITACHI Model 200-20 Spectrometer and by a HITACHI R-22 NMR Spectrometer, respectively. <sup>13</sup>C-NMR spectra were kindly taken by JNM FX-100 FT-NMR Spectrometer of JEOL Ltd. IR spectra of DHPs were measured by JASCO Model IR-S with KBr disks (1 mg sample/200 mg KBr).

##### 2.5.3 Gas chromatography

TMS derivatives of acidolysis products were analyzed by gas chromatography. Column: OV-17 on Chromosorb W, 2 m. Temperature: 190°C.

##### 2.5.4 Thermal softening

Thermal softening behavior of the DHPs were measured using a thermomechanical analyzer (TM 1500, SINKU RIKO Co. Ltd.) as the collapse of a column of powder under a constant load of 200 mg in a heated glass capillary tube.<sup>14)</sup> The measurement was conducted over the temperature range from 20°C to 400°C at a programmed heating rate of 1°C/min.

### 3. Results and Discussion

#### 3.1 Dehydrogenative polymerization of coniferyl alcohol by dialysis membrane method

In lignification of plant cell walls, dehydrogenation of monolignols occurs not in a dilute aqueous solution which is usually used on the preparation of DHP by the "Zutropfverfahren" and "Zulaufverfahren" methods but proceeds on a matrix of

cellulose and hemicellulose catalyzed by the cell wall bound peroxidase. Lignin formed in the matrix cannot be diffused from the vicinity of peroxidase. When coniferyl alcohol and hydrogen peroxide are supplied continuously to the cell walls on which previously formed DHP and peroxidase are present together in concentrated the DHP would grow up to high molecular weight materials such as natural lignin. It is understandable that high concentrations of enzyme and intermediary DHP are necessary to be formed a highly polymerized DHP. For this purpose a cellulose dialysis tube was used in the present investigation (Fig. 1).

Coniferyl alcohol and  $H_2O_2$  can pass through membrane of the cellulose tube but peroxidase and oligolignols should be impenetrable. When the sealed cellulose dialysis tube containing a concentrated peroxidase was put into the solution of coniferyl alcohol and  $H_2O_2$ , these compounds penetrate into the tube and react with peroxidase to form phenoxy radicals. These monomeric radicals are coupled so fast each other to give oligomers inside the tube which cannot pass through the membrane to the outer solution. In fact, precipitated DHP was found only inner side of the tube. The amount of the precipitate in the tube increased in the early time of the reaction, but after 24 hr the formation of the precipitate was scarcely observed in spite of the presence of coniferyl alcohol and  $H_2O_2$  in outside solution of the dialysis tube. The reason of the stop reaction seems that the inside of the membrane was coated with the precipitate of DHP formed and penetration of substrates remarkably interfered. Then a new tube containing peroxidase was replaced in the same solution and reaction was continued. The amounts of the DHPs obtained in the first and the second tubes were 58 mg and 32 mg, respectively and total yield in the twice reactions was 30%. When the reaction was continued further using new tubes in the same solution more precipitates were produced in each time. When five tubes were put into the solution at once, total yield amounted over 50% for 48 hr. The DHP obtained by this method was almost insoluble in any solvents for lignin (e.g. dioxane, THF, acetone, methanol, ethyl acetate, acetic acid, 2% NaOH and aqueous dioxane). In highly polar solvents such as formamide, DMF, DMSO and pyridine, small parts of the DHPs were dissolved but major part swelled about three to five times of the initial volume without dissolving.

### 3.2 *Effect of enzyme concentration*

Effect of peroxidase concentration on the yield of DHPs prepared by the dialysis membrane method was investigated (Table 1). In the early time of the reaction the amount of the precipitate increased in proportion to the enzyme concentrations. However, after 48 hr the yield of DHP increased only 4.1 times (from 4.1 mg to 16.7 mg) in spite of the 200 times increase of enzyme concentration (from 0.05 mg to 10.0 mg). As one reason, the rate of supply of coniferyl alcohol seems to be limited for

the activity of enzyme. However, as main reason it seemed that the precipitate produced on the inside of the membrane during reaction interfered the penetration of coniferyl alcohol. These results are shown in Table 2. When enzyme concentration was increased twice the yield of DHP obtained increased only 1.29 times ((1)/(4)). However, a double volumes of buffer gave 1.27–1.55 times of amounts of DHP in spite of the same weight of peroxidase ((2)/(1), and (4)/(3)). These results show that the yields are closely associated with the area of the membrane which is proportion to the rate and the amount of penetrated coniferyl alcohol.

### 3.3 Comparison of DHPs prepared by dialysis membrane, Zutropfverfahren and Zulaufverfahren methods

In the dialysis membrane method, DHP obtained after 30 min reaction was

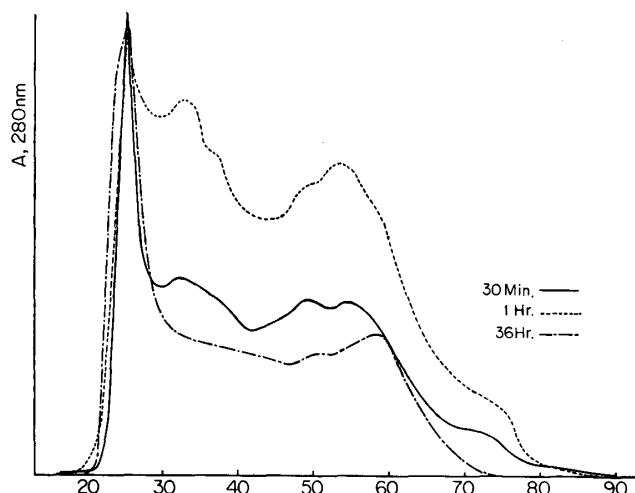


Fig. 2. Gel filtration chromatography of c-DHPs (Dialysis method) on Sephadex LH-60 with DMF

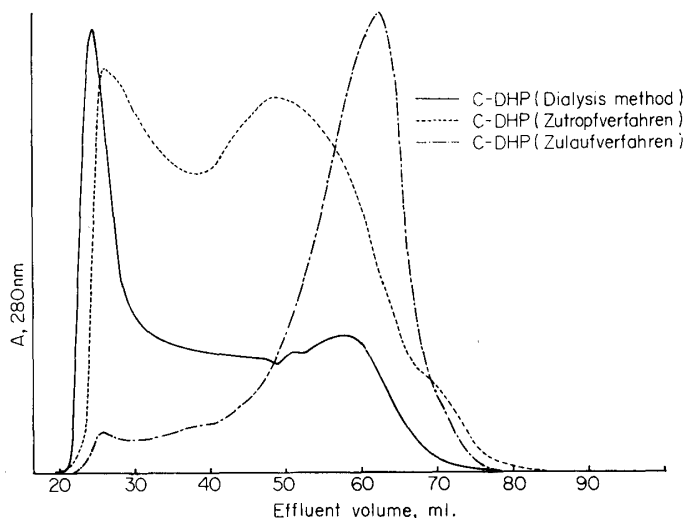


Fig. 3. Gel filtration chromatography of c-DHPs synthesized by three different methods on Sephadex LH-60 with DMF

already highly polymerized and swelled but apparently not dissolved in DMSO. Even the dissolved part, the molecular weight was higher than that by Zutropfverfahren after 36 hr reaction (Fig. 2 and 3). All dialysis method DHPs were scarcely dissolved in any solvents and therefore, the measurement of molecular distributions by gel filtration was carried only for small parts of the soluble fractions. Then molecular weight of the total DHP could not be measured by this method. However, the dialysis method DHP seems to be a very high polymer and the molecular weight may be close to that of natural lignin. The molecular weight of the Zutropfverfahren DHP increased progressively in the early time as the reaction time was longer but after 12 hr the increase of molecular weight was depressed. For the Zulaufverfahren DHP the lower molecular fractions of monomer and dimer decreased rapidly but oligomers fraction scarcely shifted to the higher molecular weight ones.

All of the DHPs obtained by Zutropf and Zulauf methods dissolved in dioxane, DMSO, etc., whereas the dialysis method DHPs were very high polymers and their solubilities were very low even in the DHP obtained in the early time of the reaction.

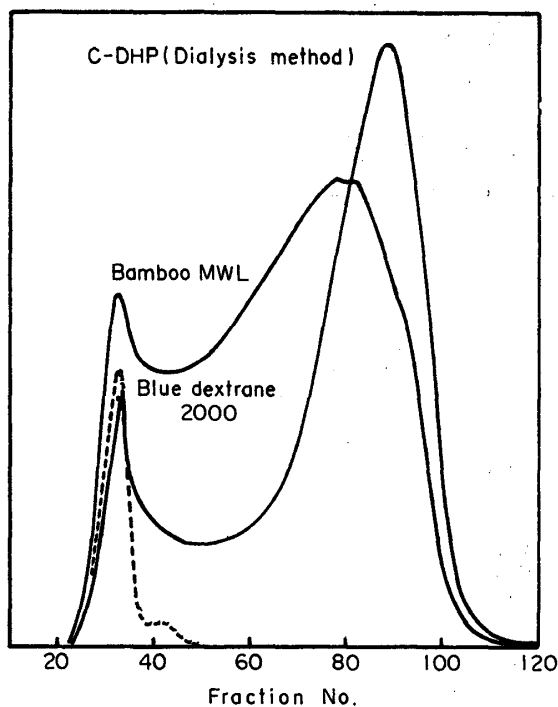


Fig. 4. Gel filtration chromatography of a soluble portion of c-DHP (Dialysis method) and Bamboo MWL on Sephadex G-200 with formamide

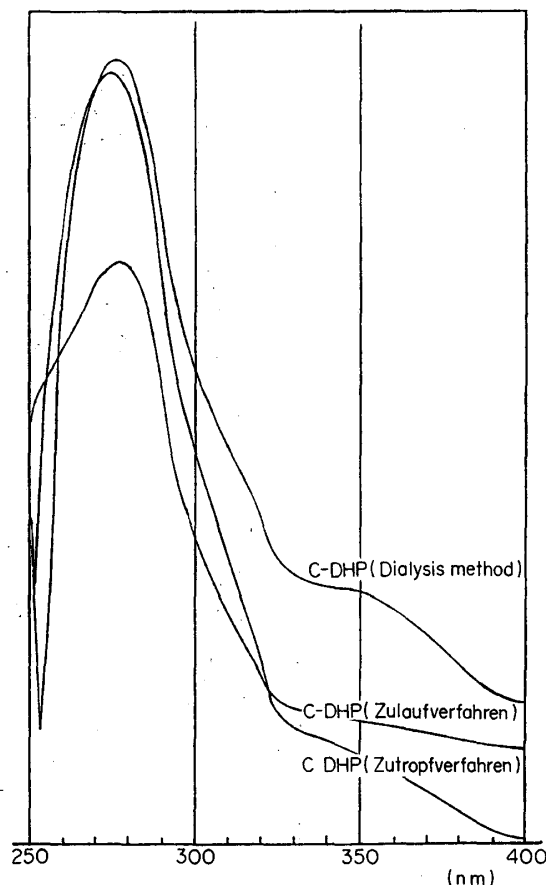


Fig. 5. UV spectra of c-DHPs by three synthetic methods



The high molecular weight and insoluble fractions increased with increasing reaction time.

### 3.4 Properties of the dialysis membrane method DHPs

Molecular weight distribution of the whole DHP obtained by dialysis method could not be measured by gel filtration and other usually used methods in the field

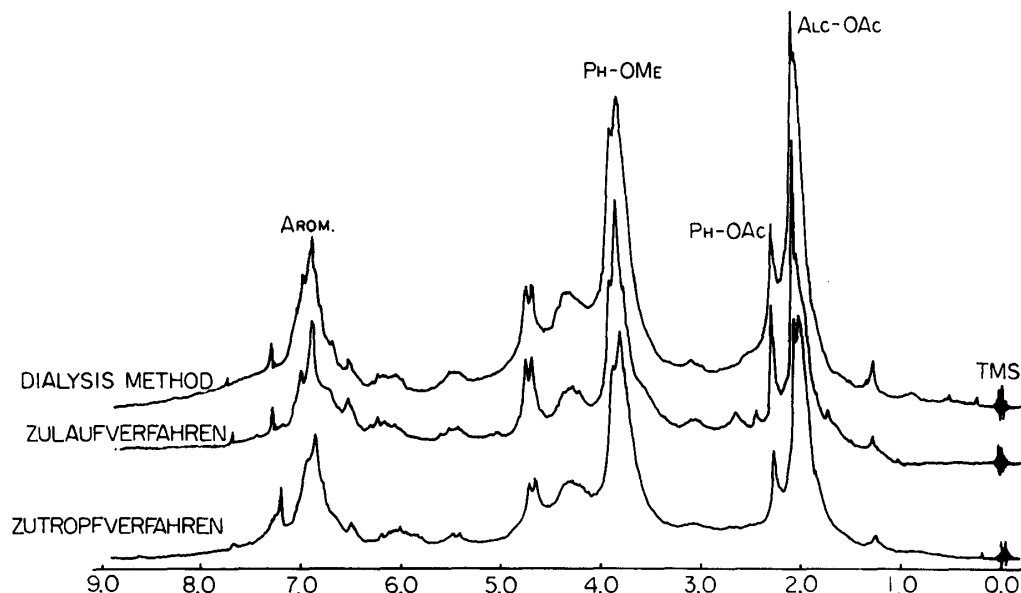


Fig. 6. NMR spectra of acetyl c-DHPs synthesized by three different methods (Zulaufverfahren, Zutropfverfahren, and Dialysis method)

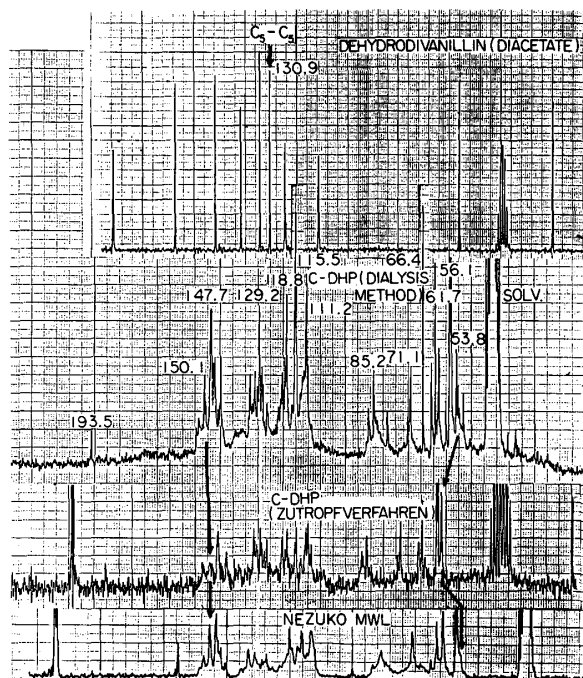


Fig. 7.  $^{13}\text{C}$ -NMR spectra of c-DHPs and MWL

of lignin chemistry because of their insolubilities. However, much larger molecular weight of the DHP than that of MWL would be presumed in view of the molecular distribution for the soluble fraction of dialysis method DHP (Fig. 4).

UV, NMR and  $^{13}\text{C}$ -NMR spectra of the soluble fraction of the dialysis method

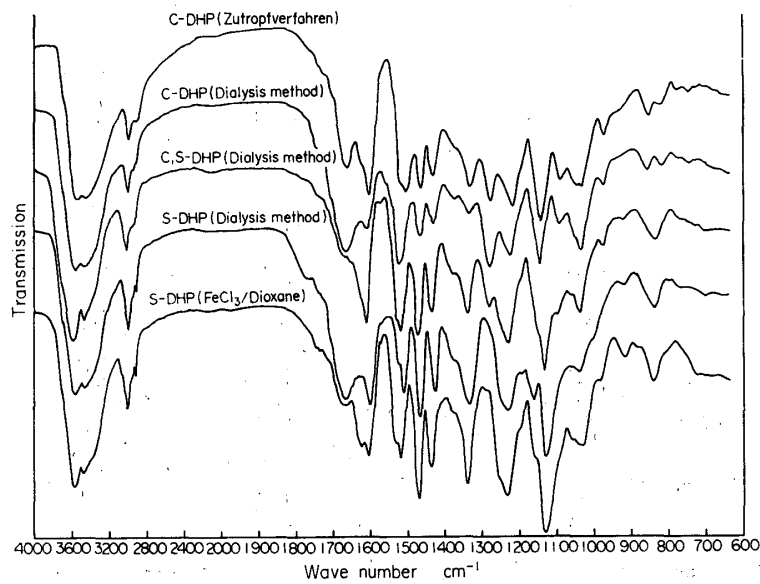


Fig. 8. IR spectra of c-DHP, c,s-DHP synthesized by the different methods

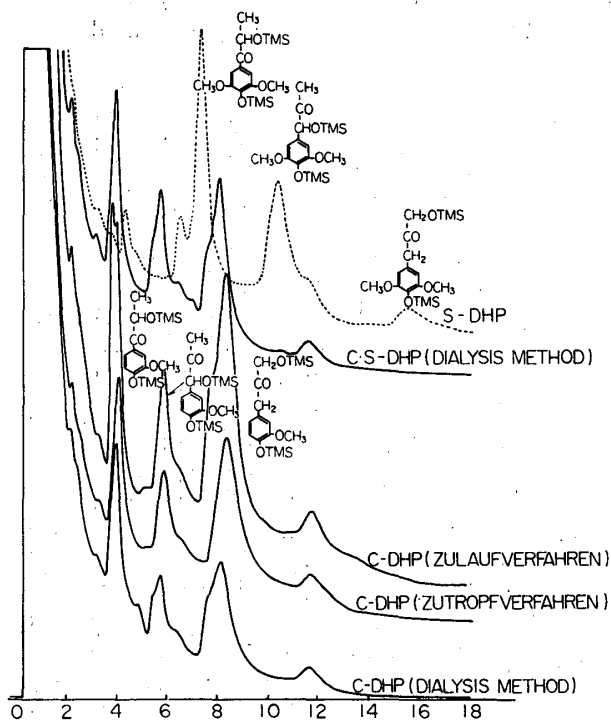


Fig. 9. Gas chromatograms of TMS derivatives of acidolysis products from DHPs

DHP and IR spectrum of the total DHP were mostly similar to those of Zutropf-DHP and bamboo MWL (Fig. 5-8). The dialysis method DHP gave typical acidolysis monomers as in the case of Zutropf-DHP and bamboo MWL.

However, slight differences are found among these polymers; shoulders at 310 and 340 nm in UV and a comparatively strong IR absorption due to conjugated carbonyl groups were found in the dialysis method DHP. Other slight differences shown in  $^{13}\text{C}$ -NMR are probably due to decrease of resinol structure (53.8 ppm) and increases of biphenyl (129.2 ppm) and  $\alpha$ -carbonyl (193.5 ppm and 150.1 ppm), which may be in agreement with high polymerization of the dialysis method DHP.

The occurrence of large amounts of carbonyl groups in the dialysis method DHP indicate the more oxidation occurred during DHP formation by this method. It is presumed that polymerized DHP in the dialysis tube was oxidized by peroxidase, and that the oxidation rate was faster than the penetration rate of coniferyl alcohol into the dialysis tube. When the dehydrogenative polymerization of coniferyl alcohol was made in concentrated solution of carbohydrates the DHP obtained gave lesser amount of carbonyl groups suggesting that the carbohydrates added reacted with quinonemethides to form lignin-carbohydrate complexes (LCC) and inhibited the formation of conjugated carbonyl groups<sup>15)</sup>. Therefore, the LCC obtained by the dialysis method seems to be closely related to natural lignin.

### 3.5 Preparation of sinapyl alcohol DHP

DHP obtained from a mixture of coniferyl and sinapyl alcohol by the dialysis membrane method (c·s-DHP (Dialysis method)) was also almost insoluble in any solvents and swelled in DMSO and pyridine (Fig. 10). Freudenberg reported that a mixture of coniferyl and sinapyl alcohols in equal amounts is enzymically dehydro-

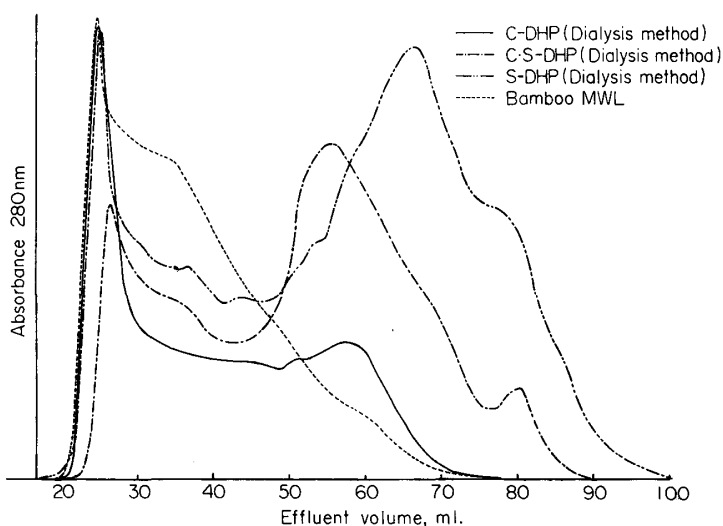


Fig. 10. Gel filtration chromatography of c-DHP, c,s-DHP and s-DHP by Sephadex LH-60 (solvent: DMF)

generated to a polymer similar to angiosperm lignin, but sinapyl alcohol alone does not give a lignin-like polymer but yields mainly syringaresinol and dimethoxybenzoquinone<sup>16)</sup>. He suggested that the steric hindrance of two methoxyl groups of sinapyl alcohol inhibits  $\beta$ -O-4 coupling and results in  $\beta$ - $\beta$  coupling to produce syringaresinol, and that no formation of syringyl lignin occurs in nature. Unexpectedly DHP was given from sinapyl alcohol alone by dialysis membrane method and obtained s-DHP (Dialysis method) was also scarcely dissolved in any solvents. The possibilities of occurrence of syringyl lignin in nature have been pointed out by Goring<sup>17)</sup>, Yamasaki et al.<sup>18)</sup> and Tanahashi et al.<sup>19)</sup>. In practice, Yamasaki et al.<sup>20,21)</sup> synthesized s-DHP by Zutropf method with very slow dropping rate. The molecular weight of Yamasaki's s-DHP (Zutropfverfahren) was about 1200 and it was soluble in dioxane and several other lignins olvents.

### 3.6 Measurement of thermal softening behavior

As this DHP is insoluble in any organic solvents the technic usually used in determination of molecular weight cannot be applied. Then the measurement of the physical properties was attempted to determine the molecular weight. Thermal softening behavior is known to be correlated with the macro-Brownian motion which reflects the molecular size and structure. Thermal softening behaviors of the DHPs in Table I were measured to elucidate the effect of enzyme concentration on the molecular weight of insoluble DHPs. The softening temperatures ( $T_s$ ) went up to

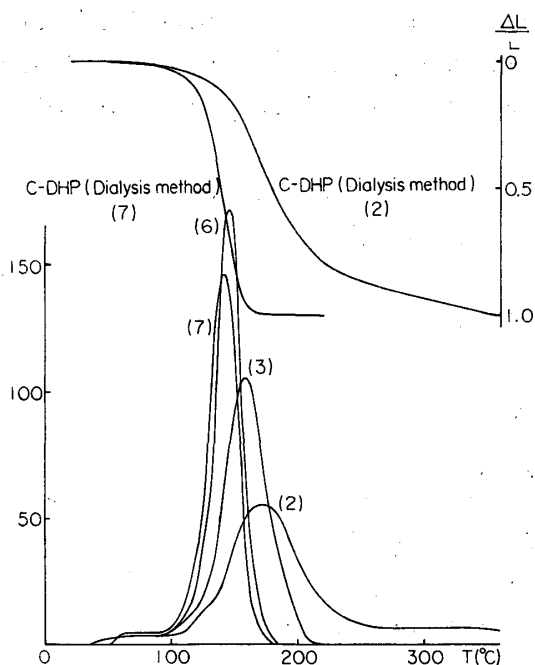


Fig. 11. Thermal softening of c-DHPs (Dialysis method)

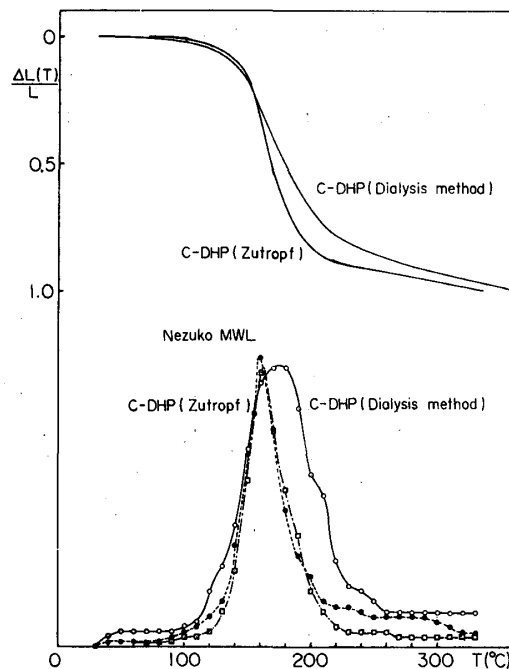


Fig. 12. Thermal softening of MWL and c-DHPs

the high temperature range as the increasing of enzyme concentration as shown in Fig. 11. MWL and c-DHP (Zutropfverfahren) showed the same Ts at 160°C whereas c-DHP (Dialysis method) showed a higher Ts at 175°C than MWL and shoulders at 205 and 230°C as shown in Fig. 12. The Ts peaks of MWL and c-DHP (Zutropfverfahren) are sharp but in c-DHP (Dialysis method) it is broad. The presumed molecular weight of the DHP from the Ts peak at 175°C was 83,000 as compared with Goring's data of dioxanelignin<sup>22)</sup>. The molecular weight of MWL calculated by the same method was about 50,000. The value of MWL seems to be larger than weight average molecular weight 11,000 obtained for spruce MWL<sup>23)</sup>. It is because the value was calculated from thermal softening using a calibration curve for dioxane lignin<sup>22)</sup>. These facts indicate that c-DHP (Dialysis method) is a highly polymerized and widely distributed material than MWL.

Thus, it would be concluded that by dialysis membrane method coniferyl and sinapyl alcohol could be dehydrogenated to highly polymerized DHPs which are insoluble in any solvents and similar to natural lignins. These DHPs show the high polymeric properties compared with Zutropf- and Zulauf method DHPs. It is expected that further investigations of reaction conditions of dialysis method and properties of DHPs obtained lead to the elucidations of high polymeric properties of lignin and processes of lignin formation.

### Acknowledgment

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