

New Preparations of Lignin Polymer Models under Conditions that Approximate Cell Wall Lignification

II. Structural Characterization of the Models by Thioacidolysis

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keywords

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Summary

The structures of novel lignin polymer models prepared from coniferin or coniferyl alcohol under conditions that approximate cell wall lignification were characterized by thioacidolysis. The linkage-type analysis by thioacidolysis indicates that the structure of these poly(lignols) approximated that of native lignin more closely than did the structure of poly(lignols) prepared by the conventional method from coniferyl alcohol. Among various possible factors affecting the structure of poly(lignols) during polymerization under the present experimental conditions, the important ones appear to be: pH of the medium in which dehydrogenative polymerization of coniferyl alcohol takes place; relative concentrations of monomer and oligomer radicals; and the carbohydrate matrix in which polymerization occurs.

Introduction

Part I of this series dealt with the preparations of new guaiacyl-type lignin polymer models from coniferin or coniferyl alcohol under conditions that approximate cell wall lignification (Terashima *et al.* 1995). The structures of these novel poly(lignols), as characterized by ¹³C NMR, approximated that of native lignin more closely than did the structure of poly(lignols) prepared by the conventional method from coniferyl alcohol (Terashima *et al.* 1995). Recent development of the novel method of thioacidolysis followed by Raney nickel desulfurization provided an accurate tool for estimation of linkage-types between monomer units (Lapierre 1993; Lapierre *et al.* 1987, 1991a; Tollier *et al.* 1991). This paper deals with the structural characterization of the novel poly(lignols) by thioacidolysis.

Materials and Methods

Details of the methods for preparation of four types of dehydrogenation polymer (DHP) were described in the previous paper (Terashima *et al.* 1995). The DHP-CFN1 was prepared from coniferin by the action of β -glucosidase, peroxidase, and hydrogen peroxide generated *in situ* through the action of oxygen and

glucose oxidase on the glucose liberated from the coniferin. Other DHPs were prepared from coniferyl alcohol using peroxidase and hydrogen peroxide by the conventional method (CAL0), or under dehydrating condition in the presence of pectin at pH 5.0 (CAL 1), or under dehydrating condition in the presence of a large amount of pectin at pH 3.5 (CAL2). The milled wood lignin (MWL) was prepared from spruce wood meal according to the procedure described by Björkman (1956).

Thioacidolysis and subsequent Raney nickel desulfurization of natural and synthetic guaiacyl lignin were performed as previously described (Lapierre *et al.* 1987, 1991a; Tollier *et al.* 1991). The identity of the various monomeric and dimeric degradation products was systematically verified by gas chromatography-mass spectroscopy (GC-MS) (Lapierre *et al.* 1991b).

Results and Discussion

Comparison of structure between new DHPs and MWL

The thioethylated monomers and the desulfurated dimeric structures are shown in Figure 1. Table 1 shows the distribution of monomeric thioacidolysis products, while Table 2 summarizes the relative yield of dimers recovered after thioacidolysis and Raney nickel desulfurization. The information concerning each class of structures is complementary to the other, and together they provide a good measure of the degree to which synthetic poly(lignols) approximate the patterns of linkage distributions that occur in native lignins; they also, thus, become valuable aids in comparisons between synthetic poly(lignols).

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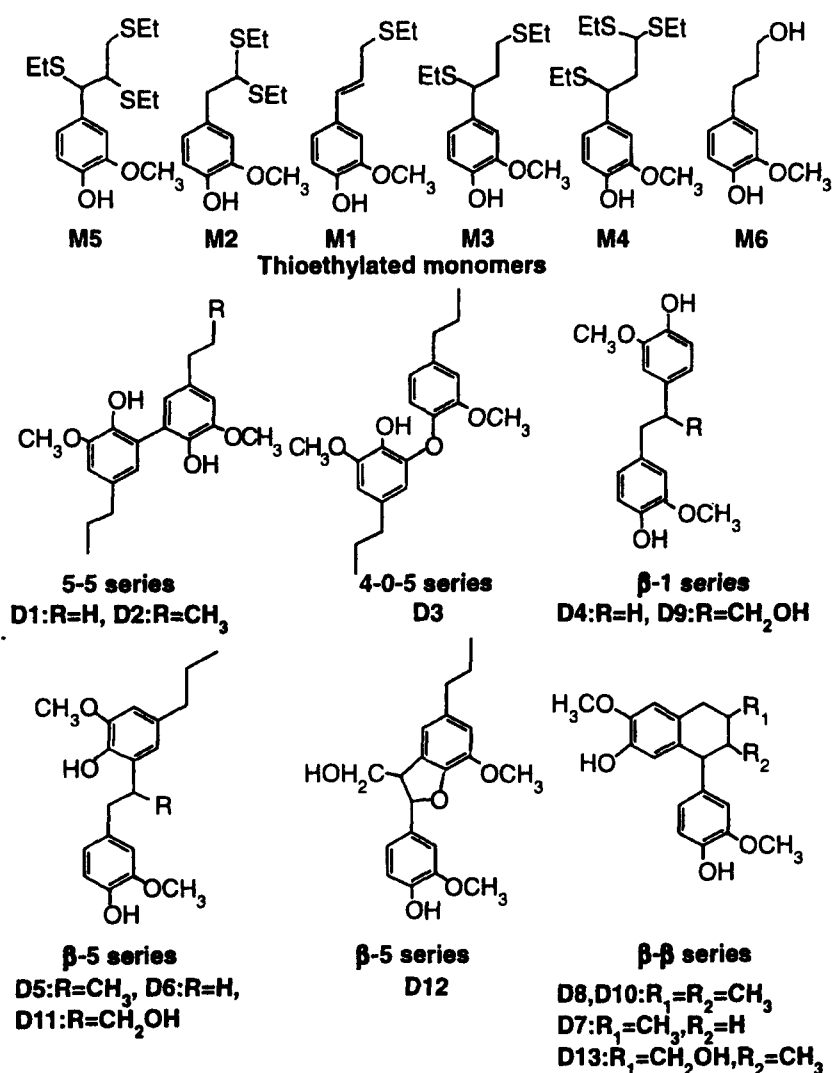


Fig. 1. Thioethylated monomers in thioacidolysis products and Raney nickel desulphurated dimers.

Table 1. Yields and relative percentages of monomeric thioacidolysis products from DHPs and lignin^a

	M5	M2	M1 + M3	M4	M6	Total
CFN1 (DHP from coniferin)	766 (71)	45 (4.2)	245 (23)	19 (1.8)	0 (0)	1075 (100)
CAL0 (DHP from coniferyl alcohol by conventional method)	621 (56)	49 (4.4)	397 (36)	19 (1.7)	22 (2.0)	1108 (100)
CAL1 (DHP from coniferylalcohol in pectin, pH 5.0)	1034 (67)	19 (1.2)	435 (28)	46 (3.0)	–	1534 (100)
CAL2 (DHP from coniferyl alcohol in pectin, pH 3.5)	810 (67)	38 (2.7)	532 (38)	34 (2.4)	–	1414 (100)
Spruce MWL	1260 (93)	–	90 (6.6)	–	–	1360 (100)

^a Structure of M1–M6 are shown in Figure 1. Values are in micromol/g of DHP or Klason spruce lignin, and values in parentheses indicate the percentage of the total products.

With respect to the distribution of inter-unit linkages, characteristic features of MWL are its high contents of β-O-4, 5-5, β-1, and 4-O-5 linkages, and low

contents of β-5 and β-β linkages in comparison with DHP as seen in Table 1 and Table 2. It should be noted that the dimeric β-5, β-β, 5-5, β-1 and 4-O-5

Table 2. Relative yield (mol%) of dimers recovered after thioacidolysis and Raney nickel desulphuration

	β -5	β - β	β -1	5-5	4-O-5
CFN1 (DHP from coniferin)	52	11	5	28	4
CAL0 (DHP from coniferyl alcohol by conventional method)	66	25	tr.	9	tr.
CAL1 (DHP from coniferyl alcohol in pectin, pH 5.0)	62	23	5.6	9	0.4
CAL2 (DHP from coniferyl alcohol in pectin, pH 3.5)	45	28	8	18	1
Spruce MWL	33	tr.	26	35	6

substructures in polyignols will give the corresponding dimeric thioacidolysis products when these substructures are bound by easily cleavable linkages (mainly β -O-4) under the conditions of thioacidolysis. If β -5 and β - β dimeric substructures are connected by 5-5 or 4-O-5 linkages in the polyignols as illustrated for the bulk polymer by Sarkanen (1971), no dimeric thioacidolysis products corresponding to β -5, β - β , 4-O-5 and 5-5 substructures will be obtained from the tetrameric substructure.

Among the thioethylated monomers shown in Figure 1, the compounds M 5 and M 2 are derived from β -O-4 structures, M 1 and M 3 from coniferyl alcohol end groups, M4 from coniferaldehyde end groups, and compound M 6 from dihydroconiferyl alcohol end groups. Analysis of the results shown in Table 1 revealed that the relative content (%) of β -O-4 type linkage, estimated from the yield of M 5 plus M 2, was higher in the DHP-CFN1 prepared from coniferin than in the DHP-CAL0 prepared from coniferyl alcohol by the conventional method and in the DHP-CAL1 and -CAL2 prepared in the presence of pectin. The relative content of β -O-4 type linkages in the CAL1 and CAL2 is also higher than that in CAL0, and closer to those of CFN1 and MWL. The content (%) of the coniferyl alcohol end groups (M1 + M3) was lower in CFN1 than those in CAL0, CAL1 and CAL2. These results indicate that the structure of the DHP from coniferin is more of an end-wise type polymer than the DHPs prepared from coniferyl alcohol. Spruce MWL contains only a small amount of coniferyl alcohol end groups (Table 1). Polyignols prepared from coniferyl alcohol contain lower levels of coniferyl alcohol end groups when glucose and glucose oxidase are used to generate hydrogen peroxide (Tollier *et al.* 1991).

The relative yields of dimeric products shown in Table 2 are also consistent with the results noted above. Thus, the contents of 5-5, β -1 and 4-O-5 substructures in CFN1 were higher than they were in CAL0, and those for β -5 and β - β were lower in CFN1 than they were in CAL0. The lower content of

β -5 and higher contents of β -1, 5-5 and 4-O-5 dimeric structures in both CAL1 and CAL2 than those in CAL0 indicate that the DHPs prepared in the presence of pectin under dehydrating conditions have structures which approximate that of MWL more closely than does the structure of conventional DHP. However, the structure of DHP from coniferin (CFN1) represents the closest approximation to that of MWL among the four DHPs.

When coniferyl alcohol was polymerized in solution using laccase and hydrogen peroxide, the main dimers found in a mixture of intermediates are β -O-4, β -5 and β - β dimers, while 5-5, β -1, and 4-O-5 dimers are not formed or are found only in very small amounts (Freudenberg and Schlüter 1955). Under the present reaction conditions using peroxidase and hydrogen peroxide, the main dimers are also the same three intermediates. The 5-5 and 4-O-5 linkages are considered to be formed mainly by reaction between dimers or oligomers, and expected to occur more frequently under the condition of bulk polymerization (Sarkanen 1971). On the other hand, under the condition of end-wise polymerization, β -O-4 and β -1 linkages are termed most frequently, and 5-5 and 4-O-5 linkages are not formed in this polymerization mode (Sarkanen 1971). The high level of both end-wise and bulk type substructures in CFN1 and MWL (Table 2) suggests that the reaction between dimers and/or oligomers which contain a high level of β -O-4 and β -1 linkages occurs more frequently under special conditions that enable both end-wise and bulk polymerization.

Factors affecting structures of polyignols

The structural characterization of the new DHPs suggested that a refinement of polymerization conditions can produce a lignin polymer model which more closely approximates native lignin than does the widely used conventional model. However, considerable differences in structure still remain between the new models and MWL. To further diminish the differences between synthetic polyignols and native

lignins, it is useful to consider the factors which may have affected the structures of the new DHPs during their preparation. Three factors possibly contributing to the modification of the structure of the DHPs during their preparation will be discussed as follows:

a) The pH of polymerization medium

Signification of differentiating cell walls is preceded by deposition of pectic substances and hemicelluloses (Terashima *et al.* 1988, 1993). This suggests that the polymerization of monolignols occurs under more or less acidic conditions. Freudenberg and Hübner (1952) prepared DHPs from coniferyl alcohol using mushroom enzyme in citrate buffer at different pH values. The UV spectrum of the DHP prepared at pH 5 was more similar to that of acetone lignin than the DHP prepared at pH 7. During the preparation of DHP-CFN1 from coniferin in the present work using β -glucosidase, glucose oxidase, peroxidase and oxygen, the pH of the reaction mixture declined to about 3.5 due to the formation of gluconic acid. In addition, DHP-CAL2 prepared from coniferyl alcohol at a pH of 3.5 seems to have structures closer to MWL than DHP-CAL1, which was prepared at a pH of 5.0 (Table 2). The low pH of the reaction medium seems to be one of the controlling factors which rendered the structures of CFN1 and CAL2 closer approximation to that of MWL. The pH may affect polymer structure at least in three ways as follows; 1) The composition of dimeric intermediates in the polymerization mixture depends on the pH (Uchida and Terashima 1990). The ratio of β -O-4 dimer to other dimeric intermediates is higher at low pH than that at high pH. 2) The distribution of diastereomers of β -O-4 depends on pH. The high *erythro to threo* ratio of β -O-4 in native lignin suggests that the pH of the medium in which lignin biosynthesis occurs is lower than has been assumed previously (Brunow *et al.* 1993). 3) Because the pH affects the activity of peroxidase, polymerization of coniferyl alcohol at low pH is slower than that at high pH. In preparation of DHP from coniferin, the activities of β -glucosidase and glucose oxidase are also affected by pH. Thus the pH affects the polymerization mode, because the concentration of coniferyl alcohol radicals depends on the activity of peroxidase as discussed in the following section.

b) Relative concentration of monomer and oligomer radicals

In the case of preparation of DHP from coniferin, the concentration of coniferyl alcohol radical is determined by the concentration of coniferin, the activities of enzymes (β -glucosidase, glucose oxidase and peroxidase), and the supply of oxygen. The supply of hydrogen peroxide is the rate-limiting factor in the

generation of reactive coniferyl alcohol radicals by peroxidase. In the preparation of DHP from coniferin, hydrogen peroxide is formed through the reduction of oxygen by glucose oxidase and glucose at the rate of its liberation from coniferin. Thus, hydrogen peroxide is supplied in an amount equimolar to coniferyl alcohol at the reaction site. Theoretically, a part of the coniferyl alcohol is incorporated into the polymer by addition to quinone methide intermediates without formation of its phenoxy radical, and 0.75 mole of hydrogen peroxide is enough to prepare DHP from one mole of coniferyl alcohol (Freudenberg 1968). In the conventional procedure for preparation of DHP, however, more than 1 mole of hydrogen peroxide has been usually added exogenously to complete the reaction. This can generate an excess of phenoxy radicals resulting in formation of a hulk type polymer. Because the volatility of coniferin is higher than that of coniferyl alcohol, the concentration of coniferin was maintained higher than that of coniferyl alcohol in the reaction mixture. If the activity of β -glucosidase is maintained at an adequate level during the reaction, coniferyl alcohol would be supplied almost constantly to a rather dense mixture of oligolignols. As discussed by Sarkanen (1971), this creates favorable conditions for more frequent reactions between monomer and oligomer radicals to produce an end-wise polymer containing more β -O-4 and β -1 structures than the reaction between two monomer radicals to give β -5 and β - β structures. The low pH of the polymerization medium may also contribute to the low radical concentration which renders the growth of polymer preferentially end-wise as discussed above.

c) Carbohydrate matrix in which polymerization occurs

It is assumed that the polymerization of monolignols in the cell wall occurs in the carbohydrate matrix because the deposition of lignin is always preceded by the deposition of polysaccharides, as shown by microautoradiography (Terashima *et al.* 1988, 1993). The deposition of lignin within the swollen gel of pectic substances and hemicelluloses reduces the hydration of the gel, resulting in a net transfer of water toward the inner undignified region together with calcium and enzymes, which are released from the reaction site as they are replaced by lignin (Terashima *et al.* 1993). The dehydration of the matrix will result in its shrinkage. This, in turn, is expected to promote more frequent reactions between oligomers resulting in increased 5-5 and 4-O-5 linkages. In the present simulation based on coniferin as precursor (CFN1), the water was removed by blowing oxygen into the reaction mixture. These conditions were favorable for reaction between oligomers. In contrast, when conif-

eryl alcohol was polymerized by the conventional procedure to prepare DHP-CAL0, oligomers usually precipitated out from the aqueous medium. Thus the low volatility of coniferyl alcohol and its oligomers in aqueous media resulted in less frequent reaction between monomer and oligomers as inferred from the low content of β -O-4 and β -1 structures in CAL0. In the preparation of DHP-CAL1 and -CAL2, water was removed continuously during the polymerization by evaporation under reduced pressure. The linkage-type analysis indicated that more frequent reaction between oligomers under the dehydrating conditions resulted in increased formation of 5-5 and 4-O-5 linkages especially in the case of CAL2 which was prepared in the presence of a large amount of pectin.

d.) *Other factors which may cause differences in structure between DHPs and MWL*

There are many other factors which may possibly cause differences in structure between DHP and MWL, among them the following: 1) Incorporation of other monolignols e.g. *p*-coumaryl alcohol and sinapyl alcohol may modify the structure of lignin though their amount is considered to be small in spruce lignin. 2) Ferulic acid esters of hemicelluloses, formed prior to lignification, may act as a lignin anchor by their participation in polymerization of lignin (Yamamoto *et al.* 1989). This may reduce the content of coniferyl alcohol end groups in MWL as seen in Table 1. 3) Enzymes other than peroxidase may participate in the cell wall lignification. Recently the participation of laccase in the early stages of lignification was demonstrated (Driouich *et al.* 1992; Sterijades *et al.* 1992, 1993; Bao *et al.* 1993), though the main enzyme responsible for the lignification in the secondary wall is considered to be peroxidase. Freudenberg and Geiger (1963) found that oxidation of the side chain of coniferyl alcohol yields coniferyl aldehyde, ferulic acid, vanillin and vanillic acid during its polymerization by mushroom enzymes, and the oxidized products are also incorporated into the poly(lignol). Thus the difference in the enzyme system for polymerization may be one of the causes for the difference in structure between DHPs and MWL. 4) Monolignols glucosides, free monolignol, and oligolignols may be associated with hemicelluloses, the orientation of which is affected by the cellulose microfibrils with which they are strongly associated (Albersheim 1975; Page 1976; Atalla *et al.* 1993; Hackney *et al.* 1994). Formation of oligolignol-carbohydrate bonds will likely increase solubility of the oligomer and promote participation in further polymerization reactions. Therefore, polymerization of monolignols and growth of the poly(lignol) molecule in the plant cell wall may proceed under sterically-restricted conditions.

Thioacidolysis as a tool for characterization of DHP

Assuming that the thioacidolysis yield is 80% for monomeric products and 60% for dimeric products (from model compound studies), and that the average molecular weight of the DHP unit is 180, the data in Table 1, together with the yield of dimeric products (not shown), indicate that about 40-50% of DHP and lignin has been characterized from the analysis of the thioacidolysis monomers and dimers. This reflects the limitation of thioacidolysis results to the degradable part of the lignin or poly(lignol) macromolecule. However, since the results of structural characterization of DHPs and MWL by thioacidolysis coincide well with those estimated by ^{13}C NMR (Terashima *et al.* 1995), which provides information on all of the solubilized portions of lignins and DHPs, the thioacidolysis can be viewed as significant confirming evidence in the study of lignin polymer models.

Conclusion

1. Four kinds of guaiacyl-type DHPs prepared under different conditions were characterized by analysis of inter-unit linkages using thioacidolysis. The results agreed with those obtained by ^{13}C NMR.
2. The structure of DHP prepared from coniferin by β -glucosidase, glucose-oxidase, peroxidase and oxygen appeared to be a closer approximation to that of MWL than that of DHP prepared by the conventional method.
3. The structure of DHPs prepared from coniferyl alcohol in the presence of pectin under dehydrating conditions also approximated that of MWL more closely than conventional DHP did, though the DHP from coniferin seems to be a better model.
4. Among possible factors controlling the structure of DHP during its formation, the pH of the reaction medium, the relative concentrations of monomer and oligomer radicals, and the carbohydrate matrix in which polymerization occurs seem to contribute to modification of the linkage type distribution.

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