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DEHYDROGENATIVE POLYMERIZATION OF CONIFERYL ALCOHOL ON MACROMOLECULAR LIGNIN TEMPLATES*

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Key Word Index—Coniferyl alcohol; dehydrogenative polymerization; lignification; lignin biosynthesis; monolignol; template polymerization; peroxidase.

Abstract—Lignins, the aromatic biopolymers present in all vascular plant cell walls, may embody as many as 10 different linkages between the constituent (*p*-hydroxyphenyl)propane residues. The final step in lignin biosynthesis has traditionally been thought to involve the *random* coupling of radical intermediates produced during the dehydrogenative polymerization of monolignol precursors. However, the enzyme-catalysed dehydropolymerization of coniferyl alcohol in homogeneous solution has now been found to be strongly affected by small quantities of macromolecular lignin components. Under these circumstances, when the radical concentrations remained low, the molecular weight distributions of the dehydropolymerisates formed with horseradish peroxidase and H_2O_2 exhibited dramatic increases in the populations of the largest species. The phenomenon was not suppressed by prior methylation of the aromatic hydroxyl groups on the causative lignin macromolecules—which, therefore, were able to promote the formation of large covalent species from the monolignol without direct participation in radical coupling. If operative in lignifying tissues, such an effect could provide the organization necessary for replicating sequences of interunit linkages along macromolecular lignin chains. In this regard, the enzyme-catalysed dehydropolymerization of coniferyl alcohol would display the character of a typical template polymerization reaction.

INTRODUCTION

Lignins, among all biopolymers, are second only to cellulose in abundance and yet little has explicitly emerged about the actual configurations of their constituent macromolecular chains. These aromatic cell wall components are formed in all vascular plants and woody tissues through the dehydrogenative polymerization of no more than three monolignols, which differ only in the methoxy substituents around the aromatic ring [1]. The precursors are believed to be sequentially incorporated into polymeric lignin structures in an order, viz. *p*-hydroxycinnamyl (*p*-coumaryl) alcohol, 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol, and 4-hydroxy-3,5-dimethoxycinnamyl (sinapyl) alcohol, which follows their positions in the biosynthetic pathway [2]. In the process they are oxidatively coupled to form as many as ten different interunit linkages, roughly half of which are the same 8-*O*-4′ alkyl aryl ether type [3].

The lignins from different plant species vary according to their predominant monomer composition. Usually lignins in softwoods (gymnosperms) are derived primarily from coniferyl alcohol, while those in hardwoods (angiosperm dicotyledons) contain similar proportions of guaiacyl and syringyl units from coniferyl and sinapyl alcohol, respectively. On the other hand, *p*-coumaryl, coniferyl and sinapyl alcohol are all incorporated to approximately the same extent into the lignins of grasses and cereals, which also contain some covalently bound *p*-coumaric and ferulic acids.

The identity of the enzyme(s) effecting the dehydrogenative coupling of monolignols to form lignins has been a matter of far-ranging debate. Peroxidase [4], peroxidase and laccase [5, 6], laccase [7, 8], (poly) phenol oxidase [9], coniferyl alcohol oxidase [10, 11] and even cytochrome oxidase [12] have been variously invoked because all have been thought to be capable of oxidizing monolignols. Nevertheless, a strong temporal correlation of lignin formation with H_2O_2 generation (and presumably peroxidase activity) has been established in both *Zinnia elegans* tissues [13] and *Pinus taeda* suspension cultures [14]. These pre-

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varications have not, however, been echoed in checkered perceptions of macromolecular lignin configuration since the coupling processes through which the biopolymer is assembled from monolignol radicals are *not* generally held to be the *direct* result of enzyme action.

It has long been known that the frequencies of interunit linkages formed during the dehydrogenative polymerization of coniferyl alcohol *in vitro* vary according to whether the monolignol is all introduced at the beginning ('Zulaufverfahren') into the enzyme containing solution or added gradually ('Zutropfverfahren') to the same system [15]. Zutropfverfahren restricts radical coupling of monomers to occurring primarily with the growing ends of polymer chains [16], a state of affairs that should reflect the situation in lignifying plant cell walls.

It has customarily been felt that more than one factor is salient in differentiating between the conditions characterizing Zutropfverfahren *in vitro* and macromolecular lignin assembly in woody tissues. *In vivo* the monolignol has been claimed to be formed from its 4-*O*-(β-D-glucopyranosyl) derivative by the action of β-glucosidase; in the presence of glucose oxidase, the glucose thereby liberated would reduce O_2 to H_2O_2 which would facilitate the subsequent peroxidase-catalysed dehydrogenative polymerization of the free monomer [2]. Furthermore, this final step in the assembly of lignins within plant cell walls is realized in the presence of cellulose, hemicelluloses and pectic substances. Indeed, the 8-8′ bond has been detected in ryegrass between lignin monomer residues and ferulate moieties that are ester-linked to α-L-arabinofuranose units in arabinoxylan [17].

A less direct connection between polysaccharide and macromolecular lignin configuration could be inferred from the observation that the frequencies of linkages to the 5-position in guaiacyl residues is increased *in vitro* when coniferyl alcohol is dehydrogenatively polymerized by peroxidase–H_2O_2 in the presence of pectin, mannan or dextran [18]. Nevertheless, the dehydropolymerisate of coniferyl alcohol produced from coniferin (its 4-*O*-(β-D-glucopyranosyl) derivative) through the consecutive actions of β-glucosidase, glucose oxidase–O_2 and peroxidase–H_2O_2 has appeared to resemble native softwood lignin more closely than that formed by direct dehydropolymerization of the monolignol through Zutropfverfahren, even when the latter is carried out in the presence of pectin [19, 20].

Be that as it may, the persistence of the notion is curious that reasonable facsimiles of native lignin biopolymers can be created in homogeneous solution through such straightforward manipulations of dehydropolymerizing conditions. Actually one entry has remained conspicuously absent from the register of potential effects that could play a significant role in determining the configuration of lignins in plant cell walls; the following question has never been addressed: do the polymeric lignin chains themselves *non*covalently exert an influence upon the course of events that constitute the final step in macromolecular lignin assembly? This intriguing possibility is the subject of the presently reported work.

RESULTS

Dehydropolymerizing conditions

The enzyme catalysed dehydropolymerization of coniferyl alcohol *in vitro* has now been examined from an entirely new perspective. Meticulously devised conditions have been employed to discover whether macromolecular lignin species can in any way affect the dehydrogenative coupling of coniferyl alcohol without direct covalent participation in the process. On account of their adequate solubilities in aqueous solutions around neutral pH, high molecular weight kraft [21] lignin components were adopted for these purposes: the configuration of the native biopolymer is well preserved in such species [22]. Horseradish peroxidase–H_2O_2 [13, 14] rather than laccase–O_2 [7, 8] was chosen to provide the requisite dehydropolymerizing conditions (*vide supra*); however, the identity of the enzyme itself was not expected to play a decisive role in controlling the modes of oxidative coupling of monolignol radicals in the presence and absence of high molecular weight lignin components.

Pre-existent macromolecular lignin components

Two high molecular weight *Pinus banksiana* kraft lignin fractions were secured by ultrafiltration as groups of species embodying characteristics representative of the native biopolymer [22]. One comprised the discrete components retained (0.6% yield) from the parent lignin preparation by a 100 000 nominal molecular weight cutoff membrane in aqueous 0.10 M NaOH; ultracentrifugal sedimentation equilibrium analysis [23] revealed a (7:3 w/w) bimodal population of species with molecular weights of 51 500 and 568 000, respectively (weight-average molecular weight = 206 000). The other was isolated from the corresponding permeate solution with a 50 000 nominal molecular weight cutoff membrane; methylation (CH_2N_2) of the retained species afforded (in 8% yield) a chloroform-soluble set of 15 400 molecular weight components.

Monolignol dehydropolymerization

A protracted Zutropfverfahren was fashioned to examine whether these lignin components are capable of exerting an effect upon the molecular weight distributions of dehydropolymerisates formed from a monolignol *in vitro*. Thus, coniferyl alcohol and H_2O_2 (molar ratio 1:1.05) were very gradually introduced at a constant rate into aqueous 20% dioxane solution (3 mM phosphate, pH 7.3) containing horseradish peroxidase (2.6 ± 0.13 unit l^{-1} toward 2,2′-azinobis-

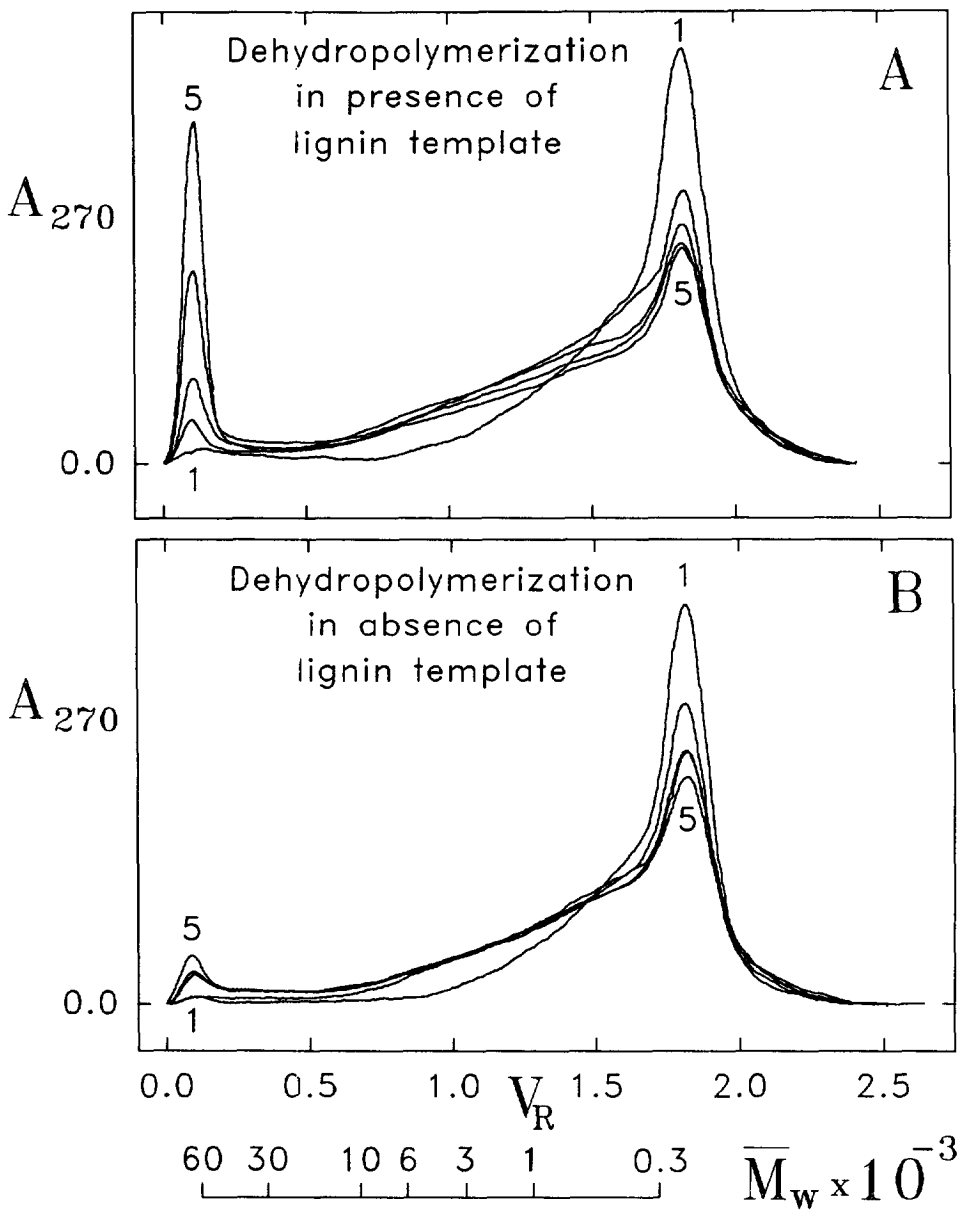

Fig. 1. Molecular weight distributions of dehydropolymerisates successively formed from coniferyl alcohol in the (A) presence and (B) absence of unmethylated macromolecular lignin template (weight-average molecular weight = 206 000, 1.0×10^{-8} M initial concentration). Zutropfverfahren introducing monolignol and H_2O_2 (1:1.05 ratio) gradually into aqueous 20% dioxane solution (pH 7.3) containing peroxidase (2.6 unit l^{-1}); total concentration of monomer units: 3.08×10^{-5} M (1) after 20 hr, 6.29×10^{-5} M (2) after 50 hr, 8.18×10^{-5} M (3) after 75 hr, 8.34×10^{-5} M (4) after 77.5 hr, and 8.50×10^{-5} M (5) after 80 hr.

(3-ethylbenzthiazoline-6-sulfonate) (ABTS) oxidation [24]) in the presence and absence of the unmethylated lignin macromolecules (weight-average molecular weight = 206 000, 1.0×10^{-8} M initial concentration) for periods of time ranging between 20 and 80 hr. The corresponding final concentrations in solution of monolignol units, both coupled and free, varied from 3.1×10^{-5} to 8.5×10^{-5} M.

The molecular weight distributions of the resulting dehydropolymerisates (Fig. 1) demonstrate that, at low concentrations in homogeneous solution, the unmethylated softwood lignin macromolecules strongly promote the formation of high molecular weight species from the peroxidase-catalysed dehydrogenative coupling of coniferyl alcohol. Indeed, the high molecular weight dehydropolymerisate components themselves seem to enhance the effect: a marked acceleration in their rate of formation (akin to autocatalysis) was observed during the later stages of the process between 75 and 80 hr [Fig. 1(A)], even though their molar concentrations remained far below those of the smaller species.

It is thus very improbable that the formation of the high molecular weight dehydropolymerisate species is

facilitated by radical coupling of the mono- (and oligo-) lignols with the (1.0×10^{-8} M) unmethylated lignin macromolecules. Indeed, an almost identical effect was evident when coniferyl alcohol and H_2O_2 (molar ratio 1:1.05) were gradually introduced into homogeneous aqueous 20% dioxane solution (3 mM phosphate at pH 7.3) where the same horseradish peroxidase activity as before (2.6 unit l^{-1}, *vide supra*) was maintained in the presence of the methylated macromolecular lignin components (15400 molecular weight, 2.7×10^{-8} M initial concentration). The final overall concentrations of the coupled and free monolignol units together again varied from 3.1×10^{-5} M after 20 hr to 8.5×10^{-5} M after 80 hr.

It is immediately apparent from the molecular weight distributions of the dehydropolymerisates (Fig. 2) that, at comparably low levels, the methylated lignin macromolecules are about as effective as the *un*methylated macromolecular lignin components (Fig. 1) in eliciting the formation of high molecular weight species during the peroxidase-catalysed dehydrogenative coupling of coniferyl alcohol. As before, an enhancement of the effect seems to be engendered by the high molecular weight dehydropolymerisate components themselves. Their rate of formation clearly underwent acceleration during the later stages of the process between 70 and 80 hr [Fig. 2(A)], even though their molar concentrations remained far below those of the smaller species.

Competition between modes of dehydropolymerization

The coupling of a coniferyl alcohol radical to another (mono- or oligolignol) radical immobilized on the surface of a macromolecular lignin component competes, of course, with the corresponding process in open solution. As the radicals proliferate when, for example, the concentration of peroxidase is made larger, coupling in open solution becomes more frequent. Such a trend is illustrated in Fig. 3(A) and (B), where the enzyme concentration has been increased 17- and 90-fold while the coniferyl alcohol and H_2O_2 were both being added 6.5 and 28 times faster, respectively, than before to the dehydropolymerizing solution. Although the methylated lignin macromolecules still tended to promote formation of the highest molecular weight entities, the proportions of middle and lower molecular weight dehydropolymerisate species rose substantially under these circumstances.

DISCUSSION

Template polymerization of coniferyl alcohol

The mechanism through which the macromolecular lignin components actuate formation of high molecular weight dehydropolymerisate species from coniferyl alcohol is likely to be governed by relatively strong nonbonded orbital interactions with the (mono- and oligolignol) radicals. The unmethylated and methylated lignin macromolecules have elicited remarkably similar effects (Figs 1 and 2) despite marked differences in their molecular weights (weight-average molecular weights of 206000 and 15400, respectively) and solubility characteristics (insoluble and soluble in chloroform, for example). This persuasively suggests that, even when radical coupling for them is absolutely precluded, lignin macromolecules can facilitate the template polymerization (*cf.* [25]) of coniferyl alcohol in homogeneous solution under peroxidase-catalysed dehydrogenative conditions.

Dehydrogenative coupling of monolignols

There are some peculiar aspects to the regioselectivity of monolignol radical coupling that, although largely taken for granted, remain poorly understood. It was reported over forty years ago [26], and recently confirmed [27, 28], that 8-5′ and 8-8′ linked dilignols are formed in larger proportions than the 8-*O*-4′ linked dimer as a result of laccase-catalysed coniferyl alcohol dehydrogenation by O_2. Somewhat later it was discovered that, during peroxidase-catalysed dehydrogenative coupling (by H_2O_2) of coniferyl alcohol with 8-5′, 8-8′ and 8-*O*-4′ linked dilignols, 8-*O*-4′ linkages predominated [29, 30], even though the proportions of the different dimers formed from the monomer alone under these circumstances are similar to those produced in the presence of laccase and O_2 [28].

As a whole, these results clearly demonstrate that the identities of the coupling products formed—from coniferyl alcohol alone and/or from coniferyl alcohol and dilignols—are not exclusively determined by the unpaired electron densities on the atomic centers involved. Otherwise, at the very least, a much larger proportion of 4-*O*-5″ linkages than observed would be expected from the dehydrogenative coupling of the monolignol with the 8-5′, 8-8′ and 8-*O*-4′ linked dimers. Thus, noncovalent interactions play an important role even in the incipient stages of coniferyl alcohol dehydropolymerization, whether or not pre-existent macromolecular lignin components are present.

Some 25 years after its first enunciation [15], Zutropfverfahren might have been consummated in the synthesis of purportedly very high molecular weight dehydropolymerisates from coniferyl alcohol by allowing the monolignol and H_2O_2 to diffuse through a cellulose dialysis membrane into contact with horseradish peroxidase at pH 6.1 [31]. However, the resulting polymeric material contained a relatively high frequency of carbonyl groups occasioned by enzyme-catalysed oxidation after dehydrogenative coupling. The intervening period had been enlivened by a report that Zutropfverfahren engenders, during the earlier stages of dehydrogenative coniferyl alcohol coupling, intermediate 'modules' with degree of polymerization 18–20 which then become linked to form

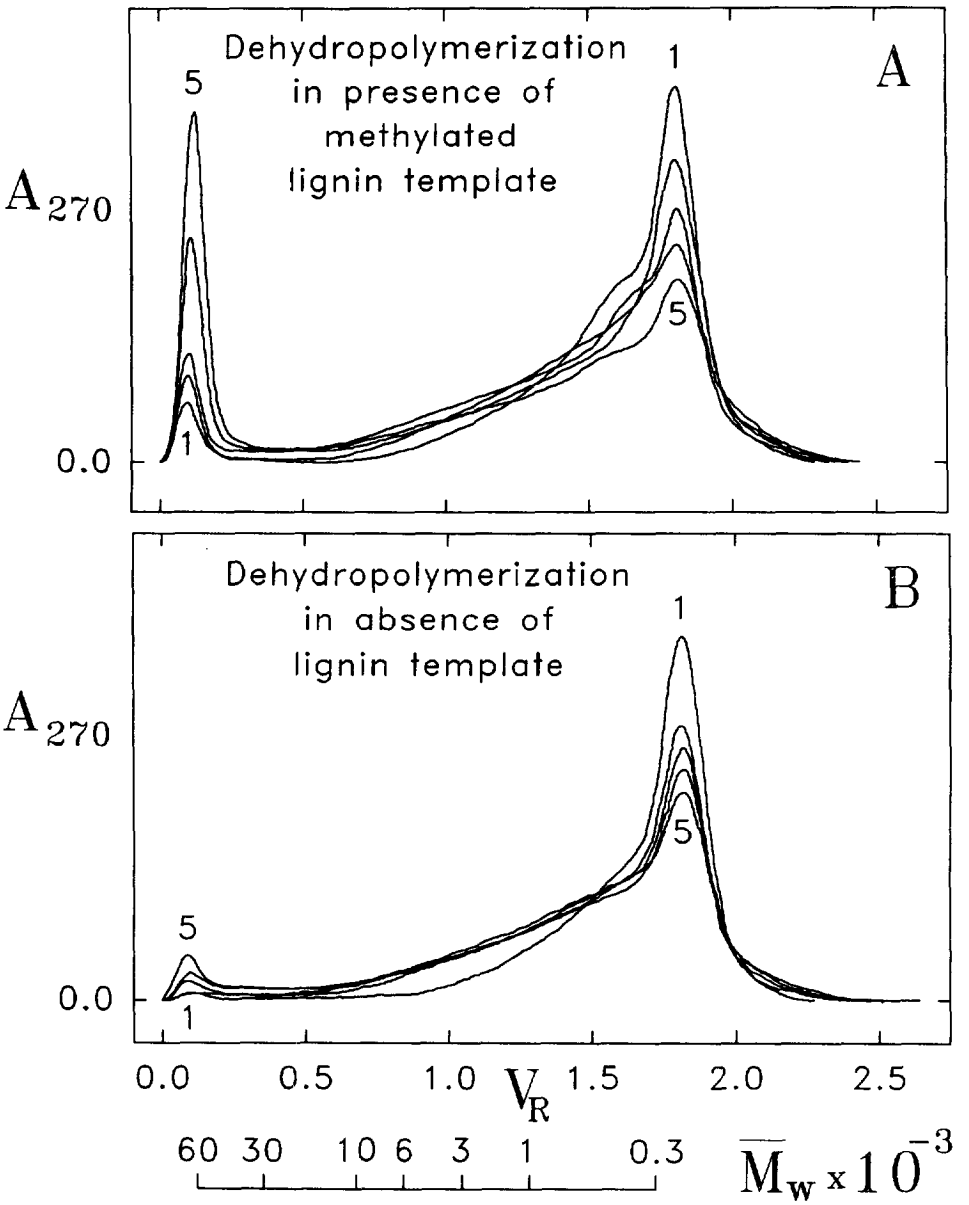

Fig. 2. Molecular weight distributions of dehydropolymerisates successively formed from coniferyl alcohol in the (A) presence and (B) absence of methylated macromolecular lignin template (15400 molecular weight, 2.7×10^{-8} M initial concentration). Zutropfverfahren introducing monolignol and H_2O_2 (1:1.05 ratio) gradually into aqueous 20% dioxane solution (pH 7.3) containing peroxidase (2.6 unit l^{-1}); total concentration of monomer units: 3.1×10^{-5} M (1) after 20 hr, 6.3×10^{-5} M (2) after 50 hr, 7.9×10^{-5} M (3) after 70 hr, 8.2×10^{-5} M (4) after 75 hr, and 8.5×10^{-5} M (5) after 80 hr.

macromolecular lignin-like species [32]. Unfortunately this claim is difficult to judge because the conditions employed for determining the molecular weight distributions of the dehydropolymerisates were conducive to extensive intermolecular association [23].

Secondary structure of lignins

It is difficult to imagine that the genesis of macromolecular lignin configurations *in vivo* could ultimately be apprehended by paying attention only to the covalent consequences of dehydrogenative mono-, oligo- and polylignol coupling in the polysaccharide matrices presented by lignifying plant cell walls [16, 19, 20]. Two sets of observations have indicated that lignins in wood cell walls possess secondary structures which are well-defined [33] in a manner that is probably established as they are biosynthesized [34]. Raman spectral analyses of *Picea marina* tracheid cross-sections with suitably polarized incident laser beams have disclosed that the aromatic rings in the lignin are, in many places, preferentially oriented in

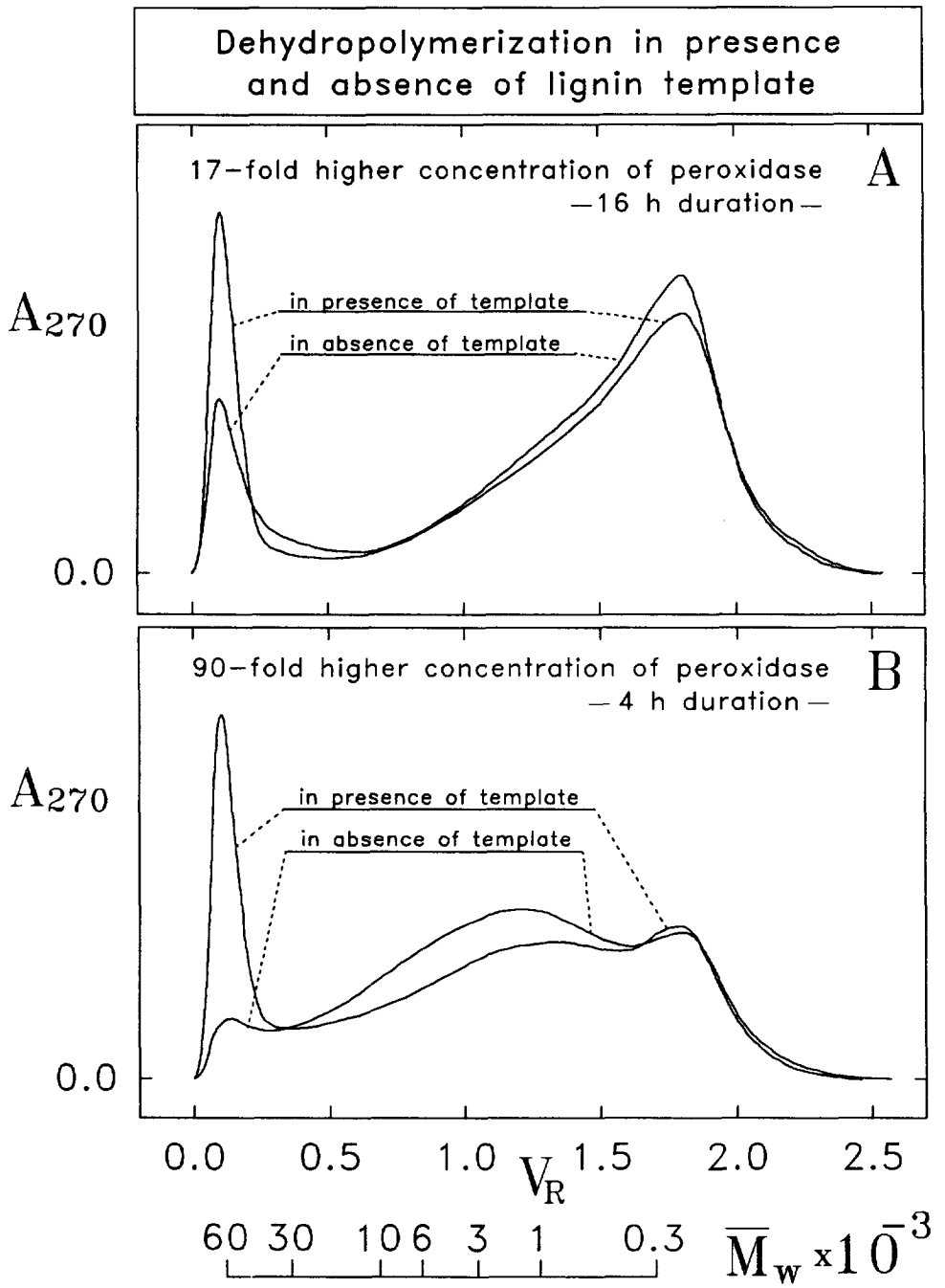

Fig. 3. Molecular weight distributions of dehydropolymerisates formed from coniferyl alcohol in the presence and absence of methylated macromolecular lignin template (15400 molecular weight, 2.7×10^{-8} M initial concentration). Zutropfverfahren introducing monolignol and H_2O_2 (1:1.05 ratio) gradually into aqueous 20% dioxane solution (pH 7.3) containing (A) 45 and 52 unit l^{-1} peroxidase, (B) 242 and 232 unit l^{-1} peroxidase, in the presence and absence, respectively, of the template; total concentration of monomer units: (A) 1.1×10^{-4} M after 16 hr and (B) 1.2×10^{-4} M after 4 hr.

the plane of the cell wall [33]. Potassium permanganate staining has suggested that compact lignin domains expand more or less uniformly in lignifying *Pinus radiata* cell walls [34]; a crosslink density of 0.052 [35] conferred by tetrafunctional branch points [36] is too low to account for such an effect without the intercession of pronounced noncovalent interactions between the macromolecular lignin chains.

Thus lignins *in situ* are characterized by a compact disposition of polymer chains in which the aromatic rings tend to be parallel to one another. Moreover, both the monomolecular film behaviour [37] and the apparent molecular dimensions visible in electron micrographs [38] have indicated that lignin derivatives are composed of disc-like macromolecular fragments cleaved from a lamellar parent structure (in the sec-

ondary wood cell wall) with thickness ~2 nm. Such arrangements of monomer residues could arise *a priori* either from the mode of dehydrogenative coupling leading to lignin formation or from conformational changes occurring after the biosynthetic events themselves. The present work strongly suggests that the former of the two alternatives, in proceeding through a template polymerization mechanism, is responsible for the secondary structure of lignins *in vivo*.

Implications for lignin biosynthesis

It is indeed highly probable that template polymerization is operative in lignifying plant cell walls since the low monolignol radical concentration prevailing *in vivo* would not give coupling a chance to occur away from the surfaces of macromolecular lignin chains. This would certainly account for the tendency towards a parallel orientation of the benzene rings observed in *P. marina* tracheids [33], whether or not it is as free radicals that monolignols first interact productively with aromatic lignin moieties. The distinct possibility arises, therefore, that characteristic sequences of inter-unit linkages in lignin macromolecules are being replicated as new polymer chains are assembled. Structures formed through post-coupling reductive processes, however, such as those in divanillyl tetrahydrofuran, dihydrodehydrodiconiferyl alcohol, shonanin and isoshonanin [14], could not be replicated directly by these means. Consequently it would be surprising if they were distributed in an incidental manner along the macromolecular lignin chains.

EXPERIMENTAL

Monolignol. Coniferyl alcohol (Sigma Chemical Co.) was purified by elution through silica gel with 10% (v/v) EtOH in petrol.

Unmethylated macromolecular lignin template. Suitable [22] discrete high molecular weight Jack pine (*P. banksiana*) kraft lignin components (0.6% yield, weight-average molecular weight = 206 000) were retained, in aq. 0.10 M NaOH during exhaustive ultrafiltration, with a 100 000 nominal molecular weight cutoff membrane (Amicon YM100) from a parent prepn isolated by acidification of black liquor produced by the Boise Cascade Corporation (International Falls, MN) [39].

Methylated macromolecular lignin template. A somewhat lower molecular weight *P. banksiana* kraft lignin fraction (5.9% yield) was similarly retained, in aq. 0.10 M NaOH during exhaustive ultrafiltration, with a 50 000 nominal molecular weight cutoff membrane (MFS Type UK) from the permeate that had first passed, under the same conditions, through a 100 000 nominal molecular weight cutoff membrane (Amicon YM100; *vide supra*). The resulting macromolecular kraft lignin fr., little altered relative to the native biopolymer [22], was methylated with CH_2N_2 (4 hr, using 23% (v/v) ethylene glycol in dioxane as solvent). After partitioning the product between chloroform and water, the fr. fully soluble in the nonaq. phase (52% yield) contained, in addition to low (< 500) molecular weight material, a set of 15 400 (±2.6%) molecular weight components (16%, based upon absorbance at 280 nm), which promoted formation of high molecular weight species during the peroxidase-catalysed dehydropolymerization of coniferyl alcohol by H_2O_2.

Absolute molecular weight determinations. Absolute molecular weights were deduced from ultracentrifugal sedimentation equilibrium analyses with a Beckman XL-A instrument. The empirical data were curve fit with sums of exponential terms describing the distributions of ideal solute components in the centrifugal field [23, 39].

Dehydrogenative polymerization of coniferyl alcohol. Three solns respectively containing 6.2×10^{-4} M coniferyl alcohol (3 mM phosphate buffer in aq. 40% dioxane), 6.5×10^{-4} M H_2O_2 (3 mM phosphate buffer in aq. 20% dioxane) and 2×10^{-3} gl^{-1} horseradish peroxidase (aq. 3 mM phosphate buffer) were simultaneously introduced gradually, each at 0.75 ml hr^{-1}, under N_2 into 256 ml (initial volume) 3 mM phosphate buffer in aq. 20% dioxane, pH 7.3, with and without 1.0×10^{-8} M unmethylated or 2.7×10^{-8} M methylated macromolecular lignin template, for periods ranging between 20 and 80 hr. The peroxidase activity (b unit l^{-1}) was monitored every ~12 hr in relation to the catalysed rate of ABTS oxidation with H_2O_2 ($b = 32.26 \times \Delta A_{405}/\Delta t$) by measuring the change of A at 405 nm (ΔA_{405}) in the time interval (Δt) between 1.0 and 6.0 min after adding substrate (1.7×10^{-3} M) and co-substrate (8.5×10^{-4} M) to aliquots (2.4 ml final vol.) of enzyme soln from the reaction flask [24]. At the end the soln containing the resulting dehydropolymerisate was freeze-dried.

Size-exclusion chromatography. Molecular weight distributions were determined by means of the corresponding elution profiles in carbonate-free aq. 0.10 M NaOH (6.5 ml cm^{-2} hr^{-1}) from Sephadex G100 in a 2.5 × 100 cm column, to which an ISCO V^4 detector was connected at the outlet. These conditions mitigate substantially the strong noncovalent interactions prevailing between lignin components in soln [23, 39]. Molecular weight calibrations were based upon ultracentrifugal sedimentation equilibrium measurements performed with a Beckman XL-A instrument on paucidisperse *P. banksiana* kraft lignin frs [23, 39].

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