Pergamon

Phytochemistry, Vol. 49, No. 5, pp. 1203–1212, 1998

Printed in Great Britain
0031–9422/98/$—see front matter

PII: S0031–9422(98)00086–7

CONSECUTIVE POLYMERIZATION AND DEPOLYMERIZATION OF KRAFT LIGNIN BY *TRAMETES CINGULATA**

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(*Received* 24 *March* 1997; *in revised form* 24 *December* 1997)

Key Word Index—Kraft lignin; laccase; lignin biodegradation; lignin depolymerase; *Trametes cingulata*; white-rot fungus.

Abstract—In extracellular solutions from white-rot fungal cultures, lignin components typically encounter opposing tendencies to be polymerized and depolymerized. The enzymes most commonly purported to bring about lignin depolymerization—lignin peroxidase, manganese-dependent peroxidase and laccase—can all act as single-electron oxidants. Accordingly they may produce from particular lignin monomer residues, either indirectly or directly, phenoxy radicals that will undergo bimolecular coupling, unless they are otherwise pre-emptively transformed. The present work has sought to elucidate whether discrimination between polymerization and depolymerization is really so precariously established in the progenitorial step of the biodegradative pathway. Some white-rot fungi express no detectable peroxidase activity of any kind and yet degrade lignins very effectively. For the sake of simplicity, one of these, *Trametes cingulata*, was selected to explore whether there might be some connection between lignin polymerization and depolymerization *in vivo*. The (Mn-free) culture medium was a multicomponent homogeneous solution containing 0.55 gl^{-1} softwood kraft lignin, which was adopted as the substrate because of its adequate solubility. As it grew vegetatively, *T. cingulata* polymerized the dissolved lignin components but then, after cessation of primary growth, the high molecular weight polymerized substrate was completely degraded. No lignin peroxidase or Mn-dependent peroxidase activity was detected in the extracellular culture solution during either metabolic phase, although (''laccase-like'') oxidase activity towards 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) persisted throughout the entire process. The results suggest that the overall effect of *T. cingulata* upon the kraft lignin substrate is governed by the consecutive release of distinct polymerizing and depolymerizing enzymes.

INTRODUCTION

Lignin depolymerization in vivo

As the second most abundant group of biopolymers, lignins in the course of their biodegradation yield a crucial step to the carbon cycle. In nature, the agents responsible for the inaugural depolymerizing transformations of lignins are produced pre-eminently by white-rot basidiomycetous fungi [1]. Apprehension of these phenomena has evolved in such an extraordinary way that an introductory summary is necessary to delineate the context which prompted the work described in the following pages.

Effect of reduced oxygen species

It was conjectured in 1980 [2] that extracellular lignin degradation in white-rot fungal cultures may not be directly mediated by enzymes but that the process could be governed by reduced oxygen species such as $O_2^{\cdot-}$ (superoxide), ·OH (hydroxyl radical), H_2O_2 (hydrogen peroxide), or even $^1O_2$ (singlet oxygen).

The idea was at first widely accepted [3–7]. It was claimed that the kinetics of H_2O_2 synthesis, but not ·OH formation, is correlated with lignin biodegradative activity in *Phanerochaete chrysosporium* cultures [6]; $O_2^{\cdot-}$ was also implicated owing to an inhibitory effect observed with superoxide dismutase on the breakdown of lignins [6].

The uncertainty about the involvement of the strongest oxidant among the reduced oxygen species was surprising since ·OH can readily be formed non-enzymatically from H_2O_2 (produced naturally in many ligninolytic white-rot fungal cultures) through transition metal ion catalyzed redox cycles. Moreover, fairly

* Paper No. 974436801 of the Scientific Journal Series of the Minnesota Agricultural Experiment Station, funded through Minnesota Agricultural Experiment Station Project No. 43-68, supported by Hatch Funds.

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complete degradation of a dehydropolymerisate from [2-^{14}C]coniferyl alcohol was achieved with 1 M H_2O_2/10 mM $FeSO_4$ [5], the customary Fenton's reagent ·OH generating system. However, the distribution of products engendered by Fenton's reagent from two dimeric nonphenolic lignin model compounds bore little resemblance to that formed in intact *P. chrysosporium* cultures [8]. A reprieve for ·OH as a plausible contributor to lignin depolymerization *in vivo* was ultimately granted in 1992 by its detection through the specific hydroxylation of phthalic hydrazide in white-rot fungal cultures [9].

During the intervening period, attention was chiefly focused upon the action of three enzymes, lignin peroxidase, manganese-dependent peroxidase and laccase; their direct involvement in lignin depolymerization has been challenged on very few occasions [9–11] even though prevailing interpretations of their effects on the substrate have obviously been controvertible.

Effect of lignin peroxidase

When first discovered in 1983, lignin peroxidase was reported to exhibit limited lignin-degrading activity. In concentrated extracellular *P. chrysosporium* culture solution containing H_2O_2 (0.2 mM) at pH 3.0, 22% of a [^{14}C]-methylated aqueous acetone extract from spruce (*Picea engelmanii* Parry) wood was apparently depolymerized within 1 h at 37°C [12]. However, no further reduction in degree of polymerization occurred during more extended incubation, whether with the cell-free culture medium or with purified lignin peroxidase [13]. Under the same conditions, a [^{14}C]-methylated birch (*Betula verrucosa* L.) milled wood lignin preparation seemed to undergo only 6% depolymerization [12].

On the other hand, in the presence of H_2O_2 produced by glucose oxidase (0.2 unit ml^{-1})/glucose (3 mM)/O_2 at pH 4.5, the enzymes in extracellular *P. chrysosporium* culture solution were at first declared to degrade a dehydropolymerisate from [*ring*-^{14}C]coniferyl alcohol by 10% during an 18 h period at 37°C [14], but the corresponding data were not disclosed. Six years later, however, a similar dehydropolymerisate from [*ring*-^{14}C]coniferyl alcohol was instead reported to undergo polymerization under comparable conditions [13].

Within three years after it was first detected, lignin peroxidase (0.8 unit ml^{-1}) with H_2O_2 was found to cause net polymerization of alkali-isolated straw and spruce milled wood lignins (1 mg ml^{-1}) at pH 4.0 [15]. The effect was enhanced by veratryl alcohol (a secondary metabolite synthesized *de novo* by *P. chrysosporium*), which has been variously believed to act as an electron transfer agent (mediator) [16], protect the enzyme from inactivation by excess H_2O_2 [17], facilitate turnover of the enzyme [18], and (in *P. chrysosporium* cultures) stimulate lignin peroxidase activity through induction [19].

Lignin peroxidase is capable of oxidizing appropriately alkoxy-substituted aromatic rings without free phenolic hydroxyl groups to cation radicals [20–22]; these may undergo (*inter alia*) proton elimination, addition of water and C-7–C-8 cleavage in phenylpropane skeleta. The problem is that phenolic hydroxyl groups are more readily oxidized by lignin peroxidase to the corresponding phenoxy radicals. Thus phenolic moieties formed after C-7–C-8 cleavage in nonphenolic arylglycerol β-aryl ether substructures together with the phenolic hydroxyl groups already present in lignins will be polymerized by lignin peroxidase through phenoxy radical coupling.

Consequently, it is only when phenoxy radical concentrations remain below the operational threshold for bimolecular coupling that depolymerization of a lignin preparation by lignin peroxidase could ever be anticipated. Accordingly, dehydropolymerisates from coniferyl and sinapyl alcohols [23] on the one hand, and coniferyl alcohol alone [24] on the other, have been appreciably depolymerized at 10^{-5} M monomer residue concentrations of the substrate in the presence of veratryl alcohol. Even under these limiting conditions, however, polymerization of the dehydropolymerisate from [*ring*-^{14}C]coniferyl alcohol occurred to an extent (14–28%) that was comparable to the amount (12–25%) of depolymerization observed [24] if the insoluble material formed is taken into account.

This is the closest that lignin peroxidase has come so far to acting as a lignin depolymerase. Two other enzymes have been habitually implicated in extracelluar lignin depolymerization by white-rot fungi, Mn-dependent peroxidase and laccase, but their patterns of behavior are no less troubling.

Effects of Mn-dependent peroxidase and laccase

Interest has been renewed in the old idea that phenol-oxidizing enzymes such as Mn-dependent peroxidase and laccase could catalyze lignin depolymerization. Mn-dependent peroxidase oxidizes Mn^{II} to Mn^{III} [25, 26] which may be stabilized by a chelator such as malonate [27] and act at a distance from the active center of the enzyme in accepting an electron from a phenolic lignin substructure.

In its effect upon a dehydropolymerisate from [*ring*-^{14}C]coniferyl alcohol ($\sim 10^{-3}$ M monomer residue concentration), Mn-dependent peroxidase (10^{-7} M, pH 4.5) appeared to cause both depolymerization and polymerization during the first 0.5 h, but thereafter polymerization and repolymerization predominated [28]. It was presumed that, *in vivo*, the low molecular weight components transiently formed would either be taken up by the fungus and metabolized intracellularly or be pre-emptively degraded further by lignin peroxidase. On the other hand, a dehydropolymerisate from [8-^{14}C] sinapyl alcohol (4×10^{-4} M monomer residue concentration) underwent 42% depolymerization during a 7 h period under the same

conditions [28]: in this case the second methoxyl substituent on the aromatic ring precludes radical coupling at the 5-position.

The role of laccase in lignin biodegradation is as equivocal as that of Mn-dependent peroxidase. Laccase oxidizes phenols and polyphenols to the corresponding radicals which may subsequently couple with one another; nonphenolic compounds are typically not affected [29]. Nevertheless one of three laccase fractions isolated from *Coriolus* (*Trametes*) *versicolor* has been reported to depolymerize a low molecular weight water soluble lignin preparation partially [30]. Moreover, in the presence of a substrate such as 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) presumably acting as a mediator, all three laccase fractions from *Trametes versicolor* have been capable of oxidizing nonphenolic lignin model compounds [31].

The fundamental difficulty

The question arises as to whether the progenitorial step in lignin biodegradation is really left to the activities of enzymes which, through relatively minor adjustments in solution conditions, may engender the polymerization or depolymerization (or both) of the substrate. The present work has sought to clarify the issue by enlisting the cooperation of a white-rot fungus that had been reported to degrade lignins very effectively without expressing any kind of detectable peroxidase activity [32].

Thus *Trametes cingulata* was allowed to grow in a Mn-free multicomponent homogeneous solution containing (0.55 gl^{-1}) softwood kraft lignin. When the culture conditions were adjusted in such a way that binding of the substrate to the mycelium [33] was avoided, the kraft lignin was transformed in a manner that offers a firm foundation for establishing the enzymatic basis of its polymerization and depolymerization.

Any reservations about the extensive modifications embodied in the kraft lignin relative to the native biopolymer [34] were judged to be outweighed by the potential advantages accruing to this substrate from its solubility: in open solution its accessibility to extracellular white-rot fungal enzymes would be maximized, while any capacity that it might possess for inducing [35] the ligninolytic system of *T. cingulata* could be enhanced.

RESULTS AND DISCUSSION

Culture conditions

Vigorous primary vegetative growth was manifested by *T. cingulata* in aqueous homogeneous culture solution containing 55 mM glucose, 2.4 mM NH_4^+ (as tartrate), 2.0 mM Mg^{II}, 0.68 mM Ca^{II}, 0.065 mM Fe^{II}, 12 mM phosphate (5 mM K^+ and 14 mM Na^+ as counterions), 1 ml l^{-1} trace element solution [36] *without* Mn and 1 ml l^{-1} vitamin solution [37], with 2.1 mM sulfate and 1.4 mM chloride, initially at pH 7.0. Under these conditions, the 3 mM (monomer residue concentration) softwood kraft lignin substrate did not precipitate or become bound to the fungal hyphae [33, 38] as it was transformed in the culture solution.

It had been supposed that ligninolytic activity in *P. chrysosporium* appears, irrespective of the presence of lignin, as a secondary metabolic event in response to the depletion of nitrogen as an essential nutrient [39]. This contention about the constitutive nature of ligninolytic activity was based upon a set of four (sextuplicate) experiments. The 24 h period after administration of a dehydropolymerisate from [*ring*-^{14}C]coniferyl alcohol had witnessed the release of similar $^{14}CO_2$ levels from cycloheximide-treated *P. chrysosporium* cultures that had been grown in the presence and absence, respectively, of the corresponding unlabeled synthetic lignin; the CO_2, however, accounted for only $\sim$0.3% of the total radioactivity [39].

Actually, slightly less than 1% of the same ^{14}C-labeled dehydropolymerisate had undergone much more rapid conversion to $^{14}CO_2$ than the remaining 99% in Miami silt loam soil [40]; from the Sephadex LH20/DMF elution profiles, this corresponded to the proportion of components smaller than tetramers in the synthetic lignin. Indeed, during the interval between 3 and 6 h, after introduction of the dehydropolymerisate from [*ring*-^{14}C]coniferyl alcohol to *P. chrysosporium* cultures that had been grown without the unlabeled synthetic lignin, the rate of $^{14}CO_2$ evolution exhibited a temporary plateau (the end of which demarcated $\sim$0.8% of the total radioactivity) before subsiding to a relatively constant value [39].

The identity of the rate-determining step in the overall process of lignin biodegradation is, of course, central to the correct interpretation of the foregoing results. Nevertheless, it seems that the lowest molecular weight components in the dehydropolymerisates from coniferyl alcohol can be metabolized intracellularly by *P. chrysosporium* without prior extracellular degradation. It is, however, the enzymatic basis of the latter step that is the focus of the present work.

Six years later, the presence of wheat straw dioxane lignin in *P. chrysosporium* cultures was found to enhance substantially the rate of decolorization of Remazol brilliant blue R, a dye considered suited to screening for lignin-degrading capability [35]. Moreover, under conditions where they all were very similar, the maximal rates of $^{14}CO_2$ evolution from straw lignin, after the ^{14}C-labeled substrate had been introduced, were attained in periods of time that varied inversely with the lignin concentrations already present in the *P. chrysosporium* cultures from the outset. This indicates that the lignin, or a degradation product derived from it, induces or activates a large part of the lignin-degrading system in *P. chrysosporium* even

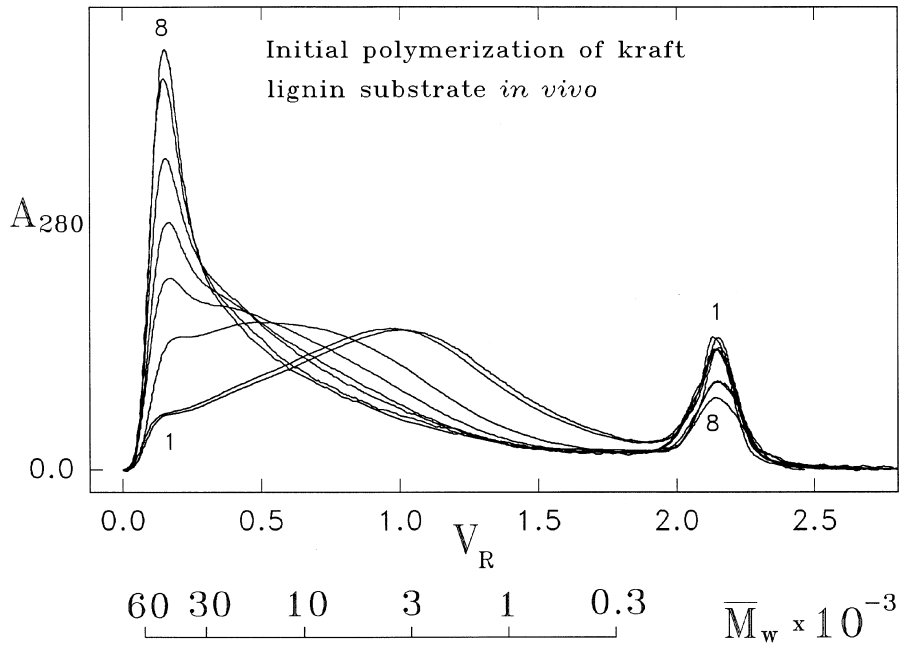

Fig. 1. Initial polymerization of softwood kraft lignin in *T. cingulata* liquid culture. Molecular weight distributions for species in extracellular solution (1) 24 h, (2) 96 h, (3) 154 h, (4) 178 h, (5) 202 h, (6) 226 h, (7) 274 h and (8) 322 h after inoculation. (Sephadex G100/aqueous 0.10 M NaOH elution profiles.)

though a low level of ligninolytic activity was preserved in the complete absence of the substrate [35].

The (erroneous) contention that ligninolytic activity is not inducible by the substrate was originally portrayed as being in harmony with a presumed lack of specificity in the lignin-degrading system itself [39]. The latter was deemed to be inherently nonspecific owing to the plethora of structures among the different interunit linkages in lignins. Indeed there is likely to be some generality in the prerequisite structural attributes of molecules, or at least their precursors, that can induce ligninolytic activity: both kraft lignins and ligninsulfonates prepared from dehydropolymerisates of coniferyl alcohol remain substantially metabolizable by *P. chrysosporium* [41].

Thus, the soluble kraft lignin substrate in the homogeneous culture solutions employed during the present work was expected to enhance any induction of the lignin degrading system in *T. cingulata*. Concomitantly, accessibility on the part of the lignin depolymerizing enzyme to the substrate should be improved under such circumstances.

Kraft lignin transformation during vegetative growth

As *T. cingulata* grew vegetatively in a homogeneous (nonagitated) culture solution, the dissolved kraft lignin underwent gradual polymerization (Fig. 1) as the pH fell to 3.9–4.2 within 13–25 days at ambient temperatures. Initial light (phosphate) buffering at pH 7.0 was necessary to prevent separation of the kraft lignin from solution onto the fungal hyphae. When the pH at the time of inoculation was lower (5.0–5.5), or if the culture solution was unbuffered, the kraft lignin was largely precipitated onto the hyphae but then exhibited no visual sign of further transformation.

Binding of a dehydropolymerisate from [*ring*-^{14}C]coniferyl alcohol to the mycelium of *P. chrysosporium* had previously been claimed to be a degradation-dependent process [38]; in this case, however, the synthetic lignin was gradually released during continued incubation of the cultures. On the other hand, more than 20% of a kraft lignin fraction soluble in organic solvents, upon introduction into 10 day old *P. chrysosporium* cultures, quickly became (and so remained) associated with the fungal cells strongly enough that its redissolution required 0.5 h exposure to aqueous 1 M NaOH at 70°C; the kraft lignin thus recovered from the cells had undergone some polymerization in relation to the original fraction [33].

As far as the present work is concerned, the soluble kraft lignin species in the homogeneous culture media were extensively associated macromolecular complexes of the kind that are spontaneously formed when the pH of an aqueous alkaline solution is lowered below 11 [42]. Thus polymerization of the kraft lignin, as the pH was reduced during vegetative growth of *T. cingulata*, must have involved the formation of covalent bonds between individual components which were already closely juxtaposed to one another.

Concomitantly, the solubility of the kraft lignin species was enhanced through the introduction of polar groups such as the α-carbonyl functionalities in phenolic units which were detected through a 350 nm peak in the ultraviolet spectra of basified solutions. Temporary buffering at pH 7.0 allowed, ahead of the

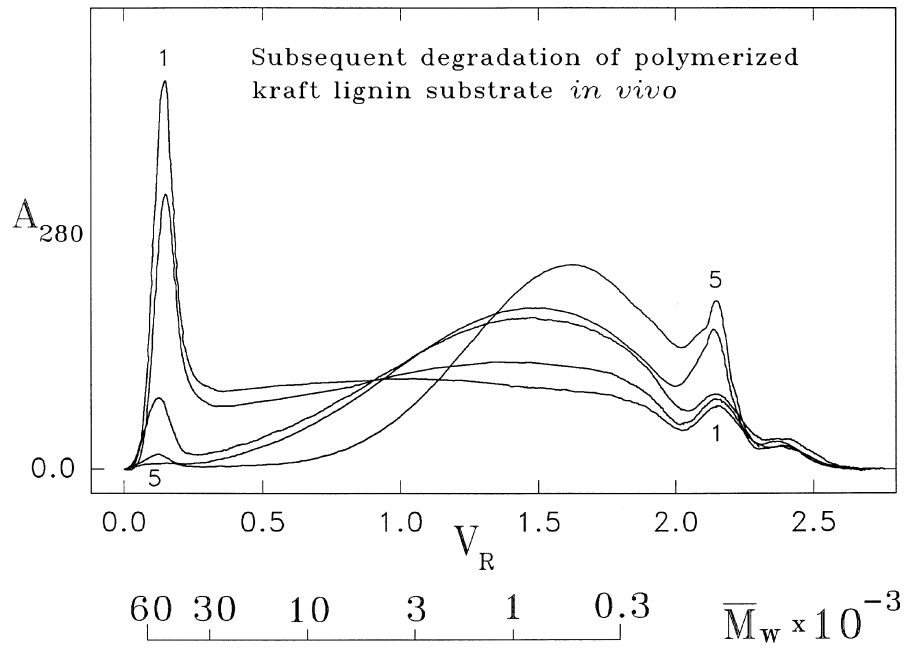

Fig. 2. Subsequent degradation of polymerized kraft lignin in *T. cingulata* liquid culture. Molecular weight distributions for species in extracellular solution (1) 5 h, (2) 149 h, (3) 329 h, (4) 353 h and (5) 509 h beyond 39 days after inoculation. (Sephadex G100/aqueous 0.10 M NaOH elution profiles.)

impending reduction in pH, an opportunity for this to occur that was sufficient to abrogate precipitation of the kraft lignin altogether. It is, moreover, interesting that selective introduction of α-carbonyl groups has been reported to accelerate the degradation of a spruce lignin preparation by *P. chrysosporium* cultures [43].

Kraft lignin depolymerization in vivo

The kraft lignin attained its maximum degree of polymerization when primary growth had ceased for *T. cingulata*. Following a brief delay, the polymerized kraft lignin, still in homogeneous culture solution, was then depolymerized during a process that witnessed the preferential and complete degradation of the high molecular weight species (Fig. 2). During this subsequent phase, the pH of the medium tended to rise somewhat (~0.3 units) to 4.2–4.5. However, in instances when the kraft lignin had precipitated onto the fungal hyphae during primary growth (if temporary buffering at pH 7.0 had not been implemented at the time of inoculation), subsequent degradation did not occur to any appreciable extent.

The Sephadex G100/aqueous 0.10 M NaOH elution profiles depicted in Figs 1 and 2 embody size-exclusion chromatographic conditions where the strong noncovalent interactions prevailing between individual kraft lignin components are largely mitigated [44, 45]. The solute species emerging from the column were monitored through their absorbance at 280 nm (A_{280}). Although the absorptivities of the kraft lignin components at this wavelength are unlikely to remain absolutely constant during the consecutive polymerizing and depolymerizing transformations of the substrate, the (appropriately scaled) areas of the elution profiles exhibited little change. Ultraviolet-absorbing (apparently extracellular) components of nonlignin origin have actually been observed in *P. chrysosporium* cultures [38]; they did substantially influence the size-exclusion chromatographic elution profiles of cell-free aqueous dioxane extracts being monitored through A_{280}, but only when the lignin concentrations in the fungal cultures were over 20-fold smaller than those employed in the present work.

Lignin preparations have been both depolymerized and (re)polymerized in white-rot fungal culture media. Extracellular culture solutions have tended to engender polymerization *in vitro* [15] while depolymerization has, of course, usually predominated *in vivo* [38]. At limitingly low $\sim 10^{-5}$ M monomer residue concentrations, dehydropolymerisates from coniferyl and sinapyl alcohols have undergone simultaneous depolymerization and polymerization in the presence of lignin peroxidase [23, 24]. The effects of Mn-dependent peroxidase on synthetic lignins have been more varied, however: a dehydropolymerisate from coniferyl alcohol ($\sim 10^{-3}$ M monomer residue concentration) was both depolymerized and (re)polymerized, while the corresponding substrate from sinapyl alcohol (4×10^{-4} M monomer residue concentration) appeared only to undergo partial depolymerization [28]. As far as the present work is concerned, the fate of the kraft lignin in *T. cingulata* cultures (Figs 1 and 2) constitutes the first case, whether *in vivo* or *in vitro*, where extensive poly-

merization of a lignin substrate has been followed by complete degradation of the resulting high molecular weight species.

Oxidative enzyme activities

Lignin peroxidase (in its compound I state) has a redox potential high enough [46] that it can effectuate single-electron oxidation of suitably alkoxy-substituted aromatic compounds without free phenolic hydroxyl groups [20–22]. Thus the enzyme is, in principle, capable of first polymerizing the phenolic components in a kraft lignin preparation and then degrading the resulting polymerized substrate. However, this subsequent degradative process could generate some new phenolic moieties that would be promptly repolymerized [47]. Consequently the pathway through which lignins in white-rot fungal cultures are extracellularly transformed, if dependent on lignin peroxidase exclusively, would be beset by opposing tendencies toward polymerization and depolymerization throughout its entire length.

The predicament which the catalytic capabilities of lignin peroxidase could engender did not ultimately arise for the transformations of the kraft lignin depicted in Figs 1 and 2: the enzyme was not excreted by *T. cingulata*. Veratryl alcohol [48] was not oxidized to veratraldehyde in the presence of H_2O_2 by the extracellular culture solutions, whether they were isolated during the polymerization or subsequent depolymerization of the kraft lignin (Table 1). The same result was, of course, obtained without the introduction of H_2O_2, confirming that veratryl alcohol oxidase [49] was also absent from the *T. cingulata* cultures. Although very slow oxidation of Azure B was observed with or without exogenous H_2O_2 in extracellular solutions isolated during the depolymerization of the polymerized kraft lignin (Table 1), the rate was not reduced in the presence of 0.01–1 mM concentrations of the putatively specific lignin peroxidase inhibitor, metavanadate [48].

Mn-dependent peroxidase was also absent during both the polymerization and subsequent depolymerization of the kraft lignin: oxidation of phenol red [48] was not detected after adding H_2O_2 and Mn^{II} to extracellular culture solutions isolated under the two circumstances (Table 1). However, vanillylidene acetone [50] was oxidized very slowly (15 μM min^{-1}) in cell-free solutions obtained from cultures witnessing depolymerization of the polymerized kraft lignin, but the same rate was observed irrespective of whether H_2O_2 and Mn^{II} had been added (Table 1).

On the other hand, ABTS was oxidized [51, 52] in extracellular *T. cingulata* culture solutions quite readily (Table 1). The rate of oxidation in (cell-free) samples of solution isolated during polymerization of the kraft lignin was 2–6 times faster than in those secured during subsequent depolymerization of the polymerized substrate (Table 1). In the latter case, ABTS oxidation at pH 5.0 was inhibited by 45% and 93%, respectively, in the presence of 0.1 mM and 0.1 M fluoride. One equivalent of fluoride can be sufficient to inhibit $\sim 10^{-4}$ fungal laccase almost completely, but a substrate may indirectly bring about a (gradual) decrease in the inhibitory effect [53].

Certainly, a typical fungal laccase appeared to be present in the *T. cingulata* culture solutions, but the data do not preclude the existence of another enzyme that might also be capable of oxidizing ABTS. This enzyme could have been responsible for the very slow rates of Azure B and vanillylidene acetone oxidation, processes that were only slightly inhibited in the presence of 0.1 M fluoride (Table 1).

From the perspective of how the kraft lignin could undergo consecutive polymerization and depolymerization in *T. cingulata* cultures (Figs 1 and 2), it might seem surprising that veratryl alcohol is not oxidized to veratraldehyde (Table 1) in the extracellular solutions isolated during the second stage of the process. Degradation of the previously polymerized kraft lignin would, *a priori*, be expected to entail oxidation of nonphenolic aromatic rings in the substrate because electron transfer from such moieties is likely to be more facile than from any other groups present. However, the fact that veratraldehyde formation was not detected does not necessarily imply that no enzyme capable of oxidizing veratryl alcohol could have been present. Thus extracellular solutions from *Lentinula edodes* cultures, when spectrophotometrically monitored at 310 nm, did not exhibit any indication of veratraldehyde formation as veratryl alcohol was being extensively oxidized to other compounds [54].

Transformations of kraft lignin substrates in vitro

In homogeneous solutions isolated from cultures supporting vegetative growth of *T. cingulata*, the kraft lignin continued to be polymerized for an extended period of time (Fig. 3). Polymerization *in vitro* occurred at an appreciably slower rate, however, than *in vivo* (Fig. 1). Cell-free samples of solution were also isolated from *T. cingulata* cultures as the polymerized kraft lignin was subsequently undergoing degradation. The depolymerizing activity did persist *in vitro*, but at a far lower level which inexorably faded completely before substantial degradation of the highest molecular weight kraft lignin species had taken place (Fig. 4).

The previously polymerized kraft lignin substrate did not undergo repolymerization *in vitro* after its degradation had ceased. In marked contrast, when newly introduced into (cell-free) culture solution isolated during degradation of the polymerized substrate *in vivo*, the original kraft lignin underwent polymerization most decisively (Fig. 5). Moreover this polymerization of the parent kraft lignin *in vitro* (Fig. 5) was much greater in extent and duration than the comparatively feeble depolymerization of the polymerized substrate (Fig. 4). Thus the premature cess-

Table 1. Oxidase activities toward different substrates in extracellular *Trametes cingulata* culture solutions

		Culture solution isolated during—					
		Polymerization of kraft lignin			Depolymerization of kraft lignin		
Substrate	Conditions and monitoring wavelength	Days after inoculation	pH*	k_{enz}[E] min^{-1}†	Days after inoculation	pH*	k_{enz}[E] min^{-1}† or μM min^{-1}‡
Veratryl alcohol (2 mM)	0.05 M tartrate, pH 3.0, 0.1 mM H_2O_2; 310 nm [48]	18	5.6	0.0	43	4.25	0.0
Veratryl alcohol (1 mM)	0.25 M tartrate, pH 5.0; 310 nm [49]	18	5.6	0.0	43	4.25	0.0
Azure B (32 μM)	0.05 M tartrate, pH 4.5, 0.1 mM H_2O_2; 651 nm§ [48]				43	4.25	0.032 μM min^{-1}¶
Phenol red (70 μM)	0.05 M malonate, pH 4.5, 0.1 mM H_2O_2, 0.2 mM Mn^{II}; 431 nm [48]	18	5.6	0.0	25	4.05	0.0
Vanillylidene acetone (0.1 mM)	0.1 M tartrate, pH 5.0, 0.05 mM H_2O_2, 0.1 mM Mn^{II}; 336 nm‖ [50]				43	4.25	15 μM min^{-1}#
ABTS (14 μM)	0.05 M glycine · HCl, pH 3.0; 436 nm [51]	18	5.6	2.1 ± 0.05 min^{-1}	25	4.05	0.37 ± 0.013 min^{-1}
ABTS (0.5 mM)	0.1 M acetate, pH 5.0; 420 nm [52]	18	5.6	2.8 ± 0.05 min^{-1}	43	4.25	1.1 ± 0.08 min^{-1}**

* pH of culture solution before adjustment.
† k_{enz}[E], where [E] is effective enzyme concentration, is equivalent to a unimolecular rate coefficient.
‡ Initial velocity of reaction.
§ Extinction coefficient at 651 nm is 4.88×10^4 M^{-1} cm^{-1} [48].
¶ No inhibition with 0.01–1.0 mM metavanadate; slight inhibition in presence of 0.1 M fluoride.
‖ Extinction coefficient at 336 nm is 1.83×10^4 M^{-1} cm^{-1} [50].
20% inhibition in presence of 0.1 M fluoride.
** 45% and 93% inhibition in the presence of 0.1 mM and 0.1 M fluoride, respectively.

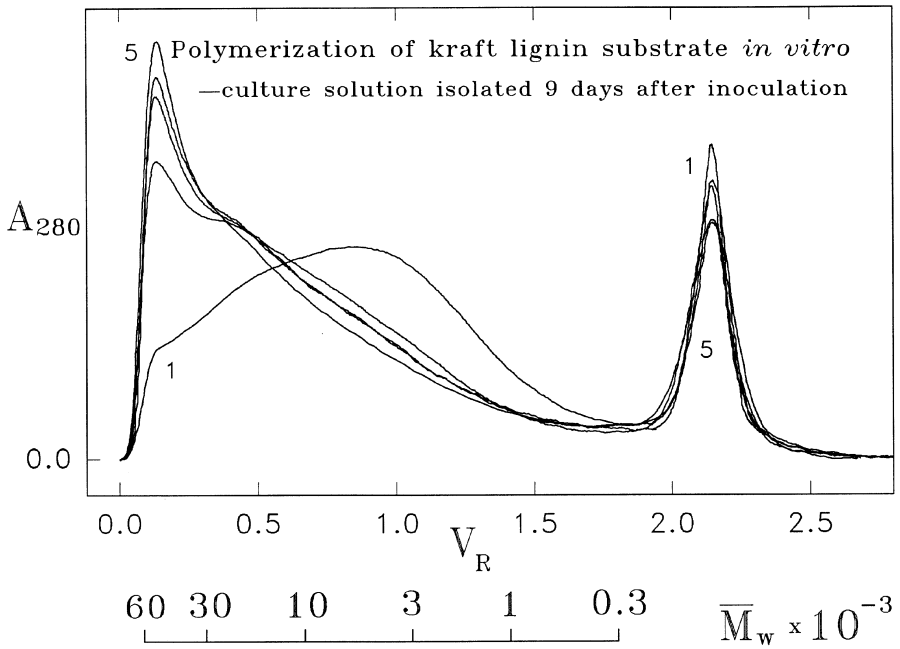

Fig. 3. Polymerization of softwood kraft lignin in (unconcentrated) *T. cingulata* culture solution at pH 4.42 *in vitro*. Molecular weight distributions for extracellular species (1) 5 h, (2) 215 h, (3) 385 h, (4) 550 h and (5) 1220 h beyond isolation 9 days after inoculation. (Sephadex G100/aqueous 0.10 M NaOH elution profiles.)

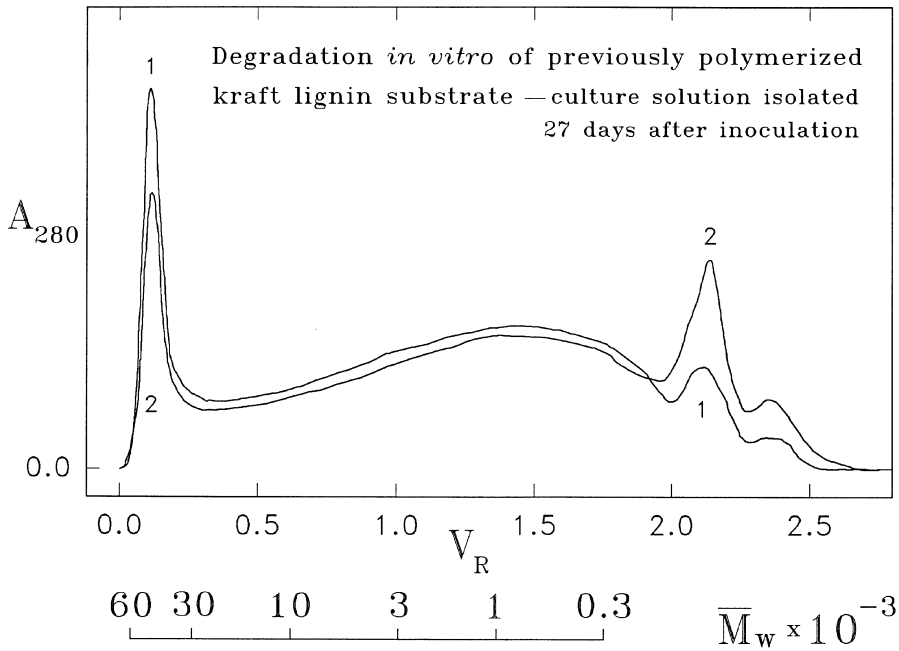

Fig. 4. Degradation of polymerized kraft lignin in (unconcentrated) *T. cingulata* culture solution at pH 4.20 *in vitro*. Molecular weight distributions for extracellular species (1) 24 h and (2) 432 h (whereafter no further depolymerization was observed) beyond isolation 27 days following inoculation. (Sephadex G100/aqueous 0.10 M NaOH elution profiles.)

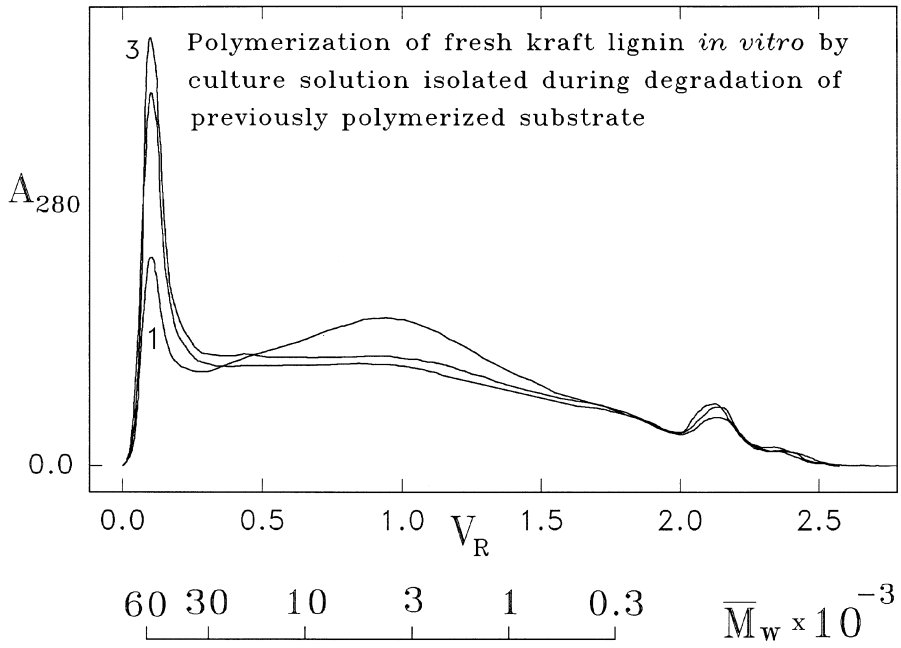

Fig. 5. Polymerization of original kraft lignin in (unconcentrated) *T. cingulata* culture solution at pH 4.25 isolated during degradation of polymerized kraft lignin. Molecular weight distributions of extracellular species (1) 96 h, (2) 384 h and (3) 960 h beyond isolation 35 days after inoculation; additional kraft lignin (0.55 gl^{-1}) was introduced 4 days after isolation of culture solution. (Sephadex G100/aqueous 0.10 M NaOH elution profiles.)

ation in the degradation of the polymerized kraft lignin *in vitro* could not have arisen from a capricious shift between opposing tendencies toward depolymerization and polymerization that were governed by the activity of a single enzyme. Rather, it was caused by the selective inactivation of the lignin depolymerase that had been released by *T. cingulata* into the culture solution.

These results indicate that the polymerization of kraft lignin and subsequent degradation of the poly-

merized substrate in *T. cingulata* cultures are caused by two distinguishable enzymatic activities. Both seem to be able to oxidize ABTS. Presumably the ABTS oxidase that exhibits the greater susceptibility to inhibition by fluoride (Table 1) is a typical fungal laccase; this enzyme is probably responsible for kraft lignin polymerization during the vegetative growth of *T. cingulata*. The other ABTS oxidase, which is not as sensitive to inhibition by fluoride, is likely to be the lignin depolymerase, but unfortunately it can lose its activity quite rapidly *in vitro*.

EXPERIMENTAL

Kraft lignin substrate

The parent kraft lignin preparation was isolated [55] by acidification of an industrial black liquor produced from Jack pine (*P. banksiana*) by the Boise Cascade Corporation (International Falls, MN).

Cultures

T. cingulata (ATCC 26747) was grown in 50 ml portions of aqueous solution (initial pH 7.0, in 500 ml conical flasks) containing 0.55 gl^{-1} parent kraft lignin, 0.244 gl^{-1} $MgSO_4$, 0.10 gl^{-1} $CaCl_2 \cdot 2H_2O$, 0.018 gl^{-1} $FeSO_4 \cdot 7H_2O$, 0.22 gl^{-1} ammonium tartrate, 10.0 gl^{-1} glucose, 0.70 gl^{-1} KH_2PO_4, 1.87 gl^{-1} $Na_2HPO_4 \cdot 7H_2O$, 1 ml l^{-1} trace element solution [36] *without* $MnSO_4$, and 1 ml l^{-1} vitamin solution [37]; these were inoculated with mycelium transferred through intervening culture solution from aqueous 1.5% malt extract (Difco). To allow air exchange, the culture flasks were fitted with (neoprene) stoppers possessing glass tubes, into each of which a glass wool plug had been loosely inserted. The stopper assemblies and flasks were autoclaved (separately) at 121°C for 30 min, while the culture solutions themselves were filter-sterilized under ambient conditions by passing through 0.2 μm Serum Acrodiscs (Gelman no. 4525). On the other hand, the malt extract solutions were autoclaved (in their flasks) for 20 min at the same elevated temperature (121°C).

Size-exclusion chromatography

Molecular weight distributions were determined by means of the corresponding elution profiles in carbonate-free aqueous 0.10 M NaOH (6 ml cm^{-2} h^{-1}) from Sephadex G100 in a 2.5×100 cm column, to which an ISCO V^4 detector was connected at the outlet. It is well established that these size-exclusion chromatographic conditions are more sensitive to covalent than to (intermolecular) noncovalent changes in kraft lignin species. Molecular weight calibrations were based upon ultracentrifugal sedimentation equilibrium measurements with a Beckman XL-A instrument, the empirical data from which were curve-fitted with sums of exponential terms describing the distributions of ideal solute components in the centrifugal field [44, 45].

Enzymatic activities in vitro

On days 9, 18, 25, 27, 35 and 43 after inoculation, suitable portions of culture solution were filtered consecutively through glass wool and 0.2 μm Serum Acrodiscs (Gelman no. 4525) with intervening centrifugation ($36{,}000 \times g$, 30 min). These solutions were used for the studies identified in Figs 3–5 and Table 1. Evidence for lignin peroxidase was sought through oxidation of veratryl alcohol at pH 3.0 and Azure B at pH 4.5, both in the presence of H_2O_2 [48], while veratryl alcohol oxidase activity was tested at pH 5.0 [49]. Indications of Mn-dependent peroxidase activity were checked by searching for the oxidation of phenol red at pH 4.5 [48] and vanillylidene acetone at pH 5.0 [50], each in the presence of H_2O_2 and Mn^{II}. ABTS oxidase ("laccase-like") activity was quite strongly displayed at pH 3.0 [51] and pH 5.0 [52].

Acknowledgements—Support of this work by the Vincent Johnson Lignin Research Fund at the University of Minnesota, the United States Department of Agriculture (Grant 91-37103-6833) and the Minnesota Agricultural Experiment Station is gratefully acknowledged.

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