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Laccases of *Rigidoporus lignosus* and *Phellinus noxius*

II. Effect of *R. lignosus* Laccase L1 on Thioglycolic Lignin of Hevea

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ABSTRACT

A laccase that has been isolated previously (*1*) from the Basidiomycete, *Rigidoporus lignosus*, a white rot fungus of rubber tree, was used in the present study. When a thioglycolic lignin (TGL) was incubated in the presence of this enzyme, pronounced changes in the UV spectrum and size distribution of the substrate were observed. Sephadex gel filtration indicated that two types of reactions occurred: (1) A degradation of the polymer, as evidenced by the production of low-molecular-weight material; and (2) a condensation of some of the TGL molecules, as revealed by an increase in the fractions of higher molecular weight.

Index Entries: Thioglycolic lignin TGL; Sephadex gel filtration and molecular weight variation estimation; molecular weight heterogeneity of TGL; laccase, enzymatic lignin depolymerization and condensation of.

INTRODUCTION

A role of phenol oxidases in the biodegradation of lignin was suggested more than 50 yr ago by Bavendamm (*2*). This author showed that white rot fungi that degraded lignin secreted a phenol oxidase, whereas

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brown rot fungi that did not degrade lignin did not produce such an enzyme. Since that time, considerable work has been carried out (*3–6*), and it was found that the specific phenol oxidase of white rot fungi was a laccase (*p*-diphenol:oxygen oxidoreductase; EC 1.10.3.2). The occurrence of both laccase excretion and lignin degradation raised the question of the effective involvement of the enzyme in lignin depolymerization.

Many reports on this aspect (*see 7–14* for reviews) have led to contradictory conclusions. Some of them (*15–21*) suggest that the enzyme catalyzes (or at least is theoretically capable of doing so) an oxidative cleavage of the macromolecule, whereas some other reports (*22–25*) conclude to a lignin polymerization activity of this enzyme. Recently, however, the latter type of activity of the enzyme has been questioned by Kern (*26*). Ishihara and Miyazaki (*16*) showed that the enzyme carried out both lignin condensation and depolymerization. Ander and Eriksson (*27*) consider the phenoloxidases (laccase and/or peroxidase) to be absolutely necessary for lignin breakdown, as well as regulating the synthesis of other enzymes involved in the degradation of the lignocellulose backbone. It has also been suggested (*28–30*) that laccase participates primarily in the detoxification of the culture medium (or of host tissues) in oxidizing phenolic compounds that are toxic for the fungus. Finally, the depolymerizing activity of laccase was not confirmed by Kaplan (*31*), who also could not demonstrate a strict correlation between the presence of the enzyme and the capacity of some microorganisms to degrade lignin (*32*).

From this brief overview of the literature, it appears that the role of fungal laccases in lignin degradation is far from being clearly determined. In previous publications (*33,34*) we reported on the excretion of laccases by the white rot fungi of Hevea roots, *Rigidoporus lignosus* and *Phellinus noxius*. One of these enzymes, Laccase L1, previously purified to homogeneity (*1*), was used to investigate its mode of action (cleavage and/or condensation) in lignin degradation by following changes in size distribution of thioglycolic lignin (TGL) used as substrate.

MATERIALS AND METHODS

Enzyme, Substrate, and Reaction Medium

Laccase L1 was purified from the culture filtrate of *R. lignosus*, as previously described (*1*). One unit of laccase is the amount of enzyme inducing an increase of the reaction medium absorbance of 0.001 OD_{420nm} in one min (in a 1-cm light-path cell), using guaiacol as substrate.

The substrate was a lignin extracted from Hevea wood by thioglycolic acid. The preliminary steps consisted of extensive extraction of a 60-mesh sawdust with the following solvents, as previously described (*35*): hot water, 0.5% ammonium oxalate, 2% sodium carbonate, alcohol-

benzene (½,v/v), and 95% alcohol. The lignin extraction process was a modified method of Holmberg (*36*). It was carried out as follows: 250 mg of extractive-free sawdust were suspended in a mixture of 0.4 mL thioglycolic acid and 5 mL HCl, 2*N*, for 5 h at 90°C. After filtration and washing, the TGL was extracted from the treated sawdust by a 2% NaOH solution. The suspension was filtered, and the filtrate was acidified. The TGL that floculates under acidic conditions was recovered by centrifugation, washed in distilled water, and dissolved in a 0.05*M* phosphate buffer at pH 6. The spectral characteristics of this solution agreed with published data (*37,38*). The absorbance of a 1% TGL solution at 280 nm was 76.2 U of OD (measured in a 1-cm light-path cell). The amounts of TGL will be expressed as total absorbance at 280 nm (1-cm light-path cell; total absorbance = $OD_{280\ nm} \times$ vol of the solution in mL), as well as mg dry material.

The enzyme reaction was carried out at room temperature (26°C), with a solution of TGL in 0.05*M* phosphate buffer at pH 6.0 (close to the pH 6.2 optimum of the catalytic activity determined in preliminary experiments), in the presence of laccase. The relative amounts of TGL and enzyme varied from one experiment to another and are indicated in the appropriate figure legends. Incubation periods varied from several hours to several days. The reaction medium was sterilized by filtering through 0.42-μm membrane filters. Controls were incubated with heat-inactivated laccase.

Analysis of Reaction Products

Determination of Molecular Weight

Changes in molecular weight (mw) resulting from the laccase action were estimated by filtration through Sephadex G-100 or G-50 in 1.8 × 100 cm columns equilibrated and eluted with 0.05*M* phosphate buffer at pH 6.0. Fractions of 1.2 mL were collected and the absorbance, at 280 nm, of the eluate was continuously recorded with an LKB Uvicord II flow-cell photometer. The apparent mw of the material in the different peaks was estimated by comparing the elution volume with that of mw marker proteins: bovine serum albumin (BSA), 68,000; ovalbumin (OVB), 43,000; carbonic anhydrase (ANH), 31,000; cytochrome *c* (CYT), 12,500, vitamin B12 (B12), 1735; and *o*- or *p*-nitrophenol (ONP or PNP), 139. In some cases the fractions were characterized by the ratio V_e/V_o (elution volume/void volume). The validity and reproducibility of gel filtration to analyze size distribution of aromatic molecules will be shown and are discussed in the Results and Discussion section.

The TGL molecules were also separated into two species with the ultrafiltration method of Sundman and Selin (*39,40*). We used an Amicon PM10 membrane whose exclusion limit was 10 kdalton in molecular mass. Fraction A was retained and contained all molecular species with a molecular mass greater than 10 kdalton. Fraction B was in the filtrate and

contained all small molecules with a molecular mass lower than 10 kdalton. Ultrafiltration was carried out in a 60-mL cell under a nitrogen pressure of 2 kg/cm^2 (30 psi) and the volume reduced to 1–2 mL. The concentrate was diluted with 50 mL of 0.05*M* phosphate buffer at pH 6.0 and concentration was run again. The successive dilutions and concentrations were repeated (6–8 times) until the last filtrate contained less than 0.5% of the initial material loaded into the cell.

Spectral Analysis

The UV-visible spectra of the substrate and reaction products were recorded between 240 and 450 nm. These determinations were performed on solutions at pH 6.0 and also on solutions of the same TGL concentration brought to pH 12 by adding concentrated sodium hydroxide. The most significant data, however, were obtained by recording the difference ionization spectrum ($\Delta\epsilon i$-curves) by reading the alkaline solution against the neutral one.

RESULTS AND DISCUSSION

Validity of Sephadex Gel Filtration as a Fractionation Method

Distribution of TGL Molecules in the Eluate

Considerable mw heterogeneity (Fig. 1) was already found with chromatography of TGL solutions on Sephadex G-100. A first class of molecules was eluted in the void volume and a second one between ap-

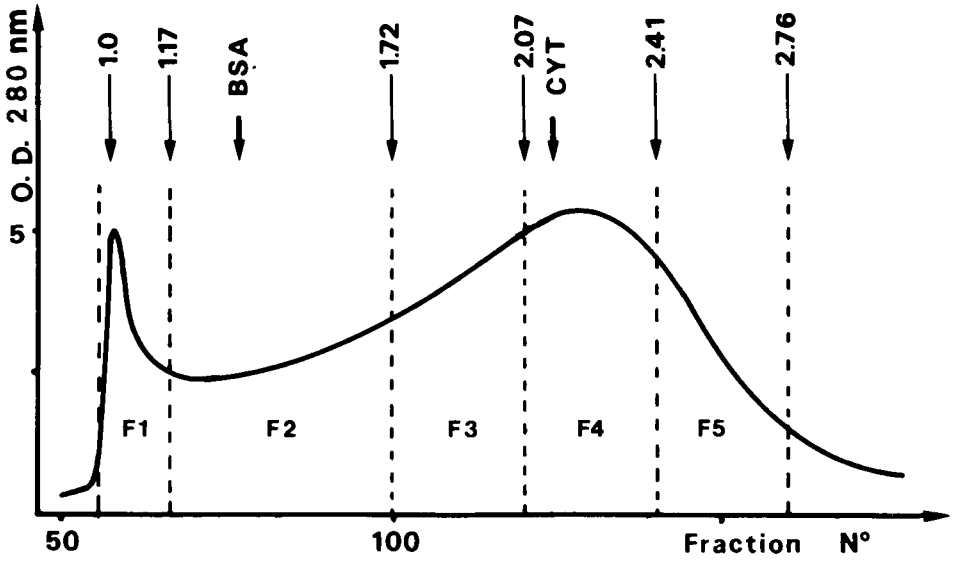

Fig. 1. Elution profile of a crude TGL solution on Sephadex G-100. Five domains, F1, F2, F3, F4, and F5, delimited by vertical dotted lines, were considered and accounted for 8.2, 22.7, 22.3, 31.9, and 14.9% of the total absorbance, respectively. The numbers above arrows represent V_e/V_0 values. For standard proteins, *see* Materials and Methods section.

parent mw values of 50,000 and 3,000, with a peak centered around 10,000. These mw values were only indicative, since the chromatographic behavior of aromatic macromolecules is not necessarily similar to that of the proteins used to calibrate the column. The overall TGL elution profile was similar to that described by Hüttermann (*41*) for the distribution of lignosulfonate molecules. Nevertheless, in the latter case, the high-mw peak accounted for more than 50% of the material chromatographed instead of the 8% found in our case (Fig. 1).

We investigated the possibility that an abnormal adsorption of the aromatic molecules on the gel disturbed the molecular sieving profiles (*42*). A solution of crude TGL was first fractionated by ultrafiltration through the PM10 membrane. The resulting concentrate A and filtrate B were then chromatographed separately on Sephadex G-100 under identical conditions. Figure 2 clearly indicates a similar behavior of the TGL molecules on the two different types of molecular sieving supports (Amicon PM10 filter and Sephadex G-100 gel).

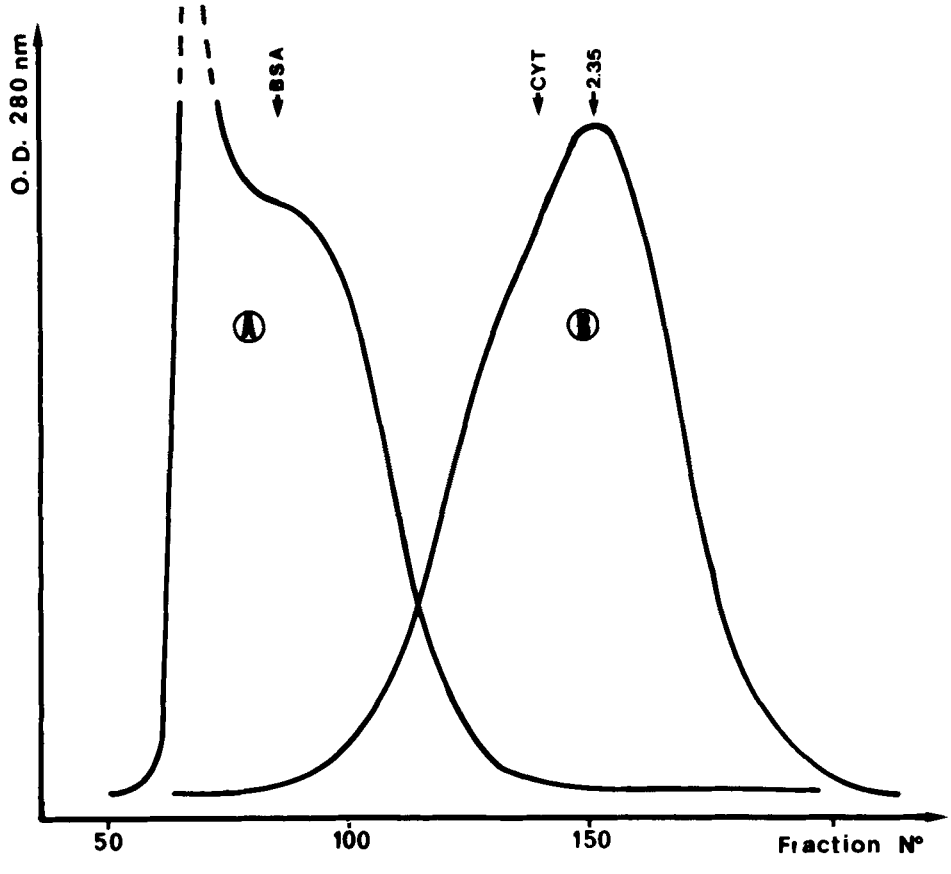

Fig. 2. Elution profiles after chromatography on Sephadex G-100 of two fractions previously separated by ultrafiltration through a PM10 membrane: (A) TGL fraction with mw higher than 10,000; and (B) TGL fraction in the ultrafiltrate with mw lower than 10,000.

Reproducibility of the Techniques

The nonreproducibility of gel filtration of aromatic molecules has been suggested by Hüttermann (*41*). A high-mw lignosulfonate fraction obtained from a first chromatography on Sephadex G-100 was rechromatographed under the same conditions and was found to split into two peaks. One of these was in the high-mw zone, the other in the zone of lower mw.

We performed similar experiments with our TGL solutions. We first chromatographed a crude TGL solution on Sephadex G-50 and collected three eluate fractions Fr1, Fr2, and Fr3, as indicated in Fig. 3A. These were then rechromatographed separately under exactly the same conditions. The results are indicated in Fig. 3B and clearly show the excellent reproducibility of the method.

Effect of Laccase on TGL

Spectral Changes

The solution rapidly turned brown as soon as the reaction began, this was accompanied by an increase in absorbance at 280 nm; this increase could reach 150% of the initial value. When compared with the difference ionization spectrum of a nonincubated TGL solution ($\Delta\epsilon i$-curves; Fig. 4A and C, curve a), the $\Delta\epsilon i$-curves (Fig. 4B and C, curve b) of a laccase-treated TGL show an attenuation of the peak at 255 nm, a strong decrease of the peak at 298 nm, and an increase of the peak at 365 nm. With reference to published data (*38*), these changes may reflect a considerable decrease in the number of ionizable phenol hydroxyl groups and an increase in the relative content of α-carbonyl groups in the polymer. To summarize, they show the oxidizing activity of laccase on TGL.

Changes in the Molecular Size of TGL

Preliminary experiments in which a crude TGL solution was incubated in the presence of laccase indicated an increase in both compounds of low and high mw. This suggested that laccase was able to both degrade and condense the TGL. To clearly distinguish these two types of activity, two size-classes of TGL substrate molecules were selected prior to incubation with the enzyme. The rationale was that depolymerization would be easier to detect by assaying substrate molecules of high mw. Conversely, a polymerization activity would be easier to evidence by incubating substrate selected for low mw.

Depolymerization Activity

Figure 5 is an unambiguous demonstration of the degradation of a high-mw TGL fraction (Fr1 type selected by molecular sieving on Sephadex G-50, shown in Fig. 3A) into several low-mw compounds. At least three peaks (I, II, and III) could be identified with respective V_e/V_o

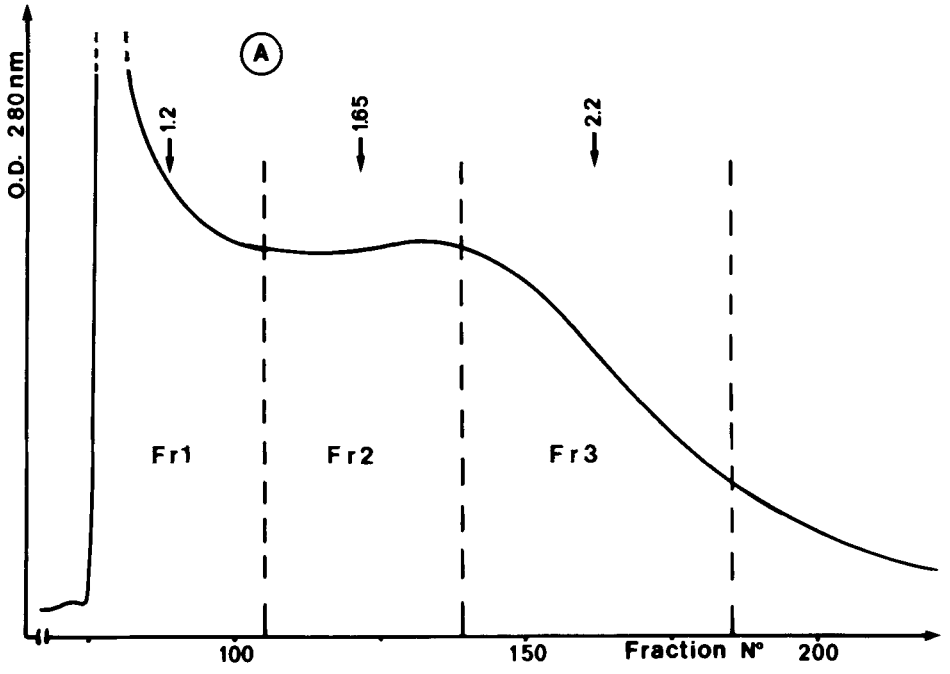

Fig. 3A. Elution profile after chromatography of a crude TGL solution on Sephadex G-50. The numbers above arrows represent V_e/V_o values. Fractions Fr1, Fr2, and Fr3 are delimited by vertical dotted lines.

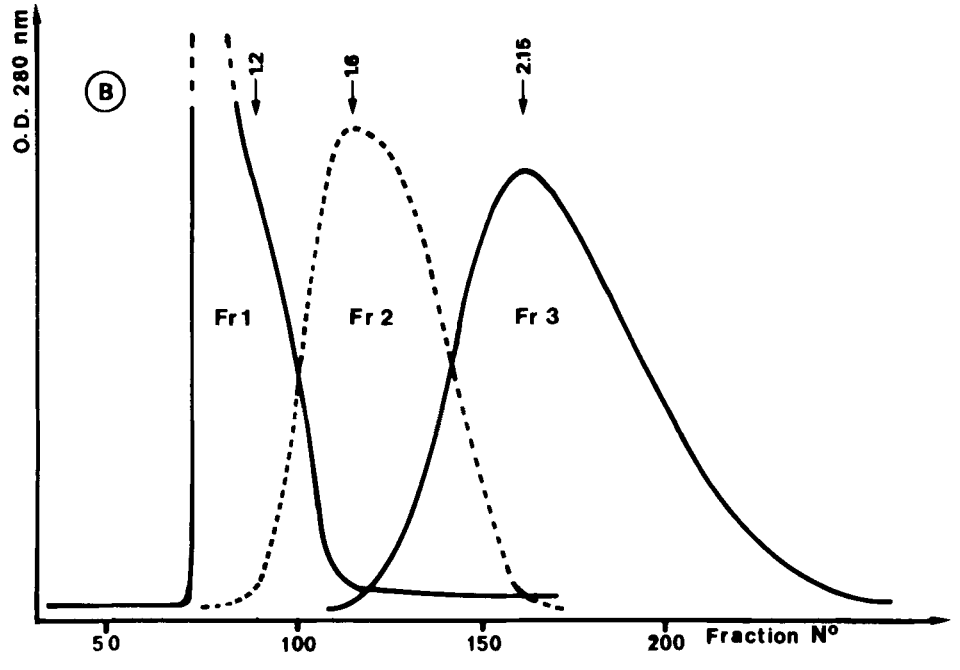

Fig. 3B. Elution profiles after rechromatography of Fr1, Fr2, and Fr3 in separate runs, but under the same conditions, on Sephadex G-50.

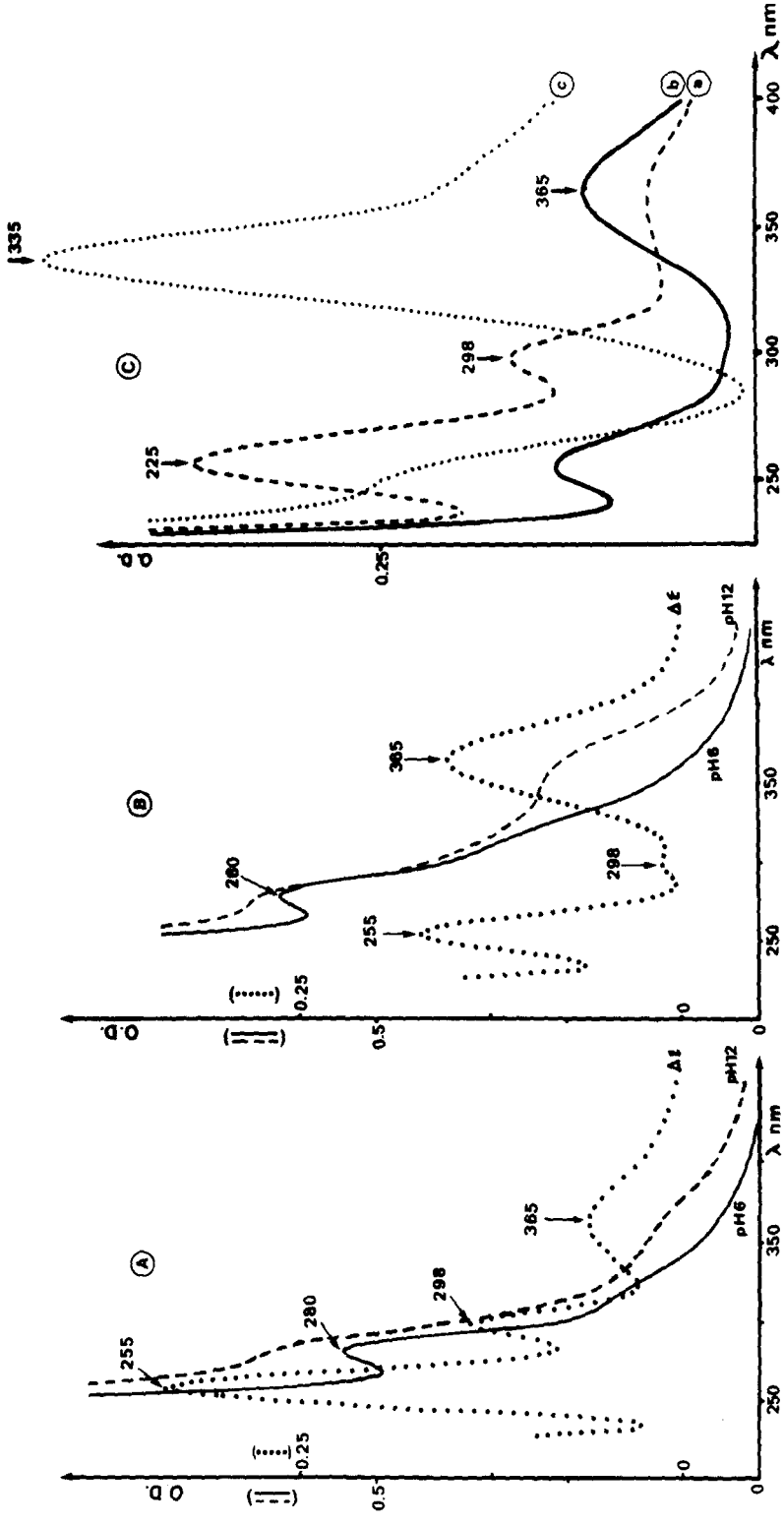

Fig. 4. The UV-visible spectra at pH 6 and 12 and difference ionization curves ($\Delta\varepsilon i$) of crude TGL solutions before (**A**) and after (**B**) incubation with the active laccase. (**C**): Comparison between different $\Delta\varepsilon i$-curves of TGL solutions before (**a**) and after (**b**) oxidation by the laccase; (c) $\Delta\varepsilon i$-curve of the degradation products isolated in fraction II after chromatography on Sephadex G-50 (*see* Fig. 5b).

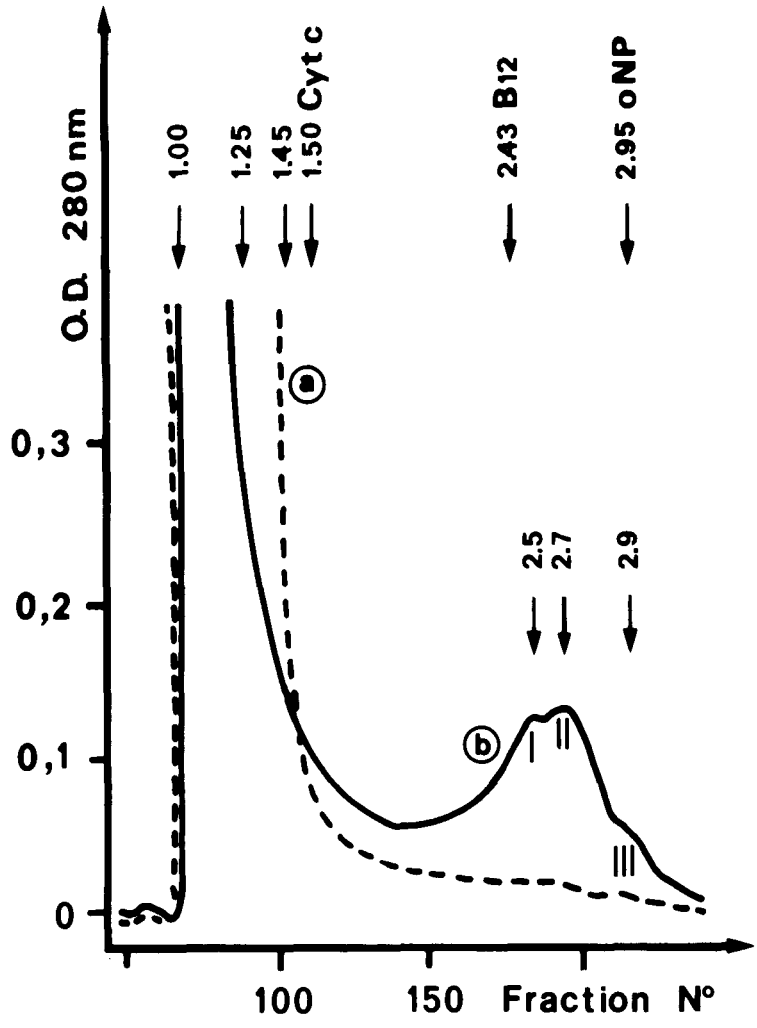

Fig. 5. Elution profile after chromatography of high mw TGL (Fr1 type, *see* Fig. 3A) on Sephadex G-50: (**a**) control (incubation with heat inactivated laccase; column load: 102 A_{280} = 13.4 mg TGL); (**b**) after a 9-d incubation with active laccase. The reaction medium contained 545 $A_{280\ nm}$ (= 71.5 mg TGL) and 3,050 U laccase in a total vol of 5 mL; the incubation resulted in a 148% increase of the initial A_{280nm} in the solution. Therefore, instead of loading the column with the same amount of TGL material as in the control experiment (a), it was loaded with an amount yielding the same total absorbance units: 101 $A_{280\ nm}$ (corresponding to 10 mg laccase-oxidized TGL). This procedure allows a well-founded comparison between the control curve (a) and the assay curve (b). Total low-mw material collected (Σ peaks I+II+III) was 6.4%.

values of 2.5, 2.7, and 2.9. With reference to the V_e/V_o values of the calibration markers, fractions I, II, and III were composed of very small molecules. They quantitatively accounted for 6.3% of the total material eluted from the column (based on total $A_{280\ nm}$).

All these fractions exhibited the spectral characteristics of oxidized TGL (Fig. 4B and curve c in Fig. 4C), except fraction II, whose $\Delta\epsilon$/-

spectrum was characterized by a peak at 335 nm and the disappearance of the peaks at 298 and 365 nm (curve c in Fig. 4C).

Polymerization Activity

Experiments in which a solution of low-mw TGL (such as fraction F4 in Fig. 1) was incubated with laccase clearly demonstrated a condensation reaction (Fig. 6). The initial peak (V_e/V_o = 2.3, curve a) was split into two classes of molecules (curve b), the first corresponding to condensation products (V_e/V_o = 1.7) and the second to apparently lower-molecular-weight compounds (V_e/V_o = 2.5). Concerning the latter class, we could not unequivocally determine whether it contained degradation products or simply residues of the original fraction not yet condensed. The $\Delta\epsilon i$-spectrum of both fractions were characteristic of oxidized TGL (Fig. 4B and curve c in Fig. 4C).

Recurrent Degradation Test

Thus, laccase apparently catalyzed both condensation and depolymerization. This could be explained in terms of either an equilibrium between the two reactions or two independent reactions involving each one particular class of molecules. We investigated the former hypothesis by

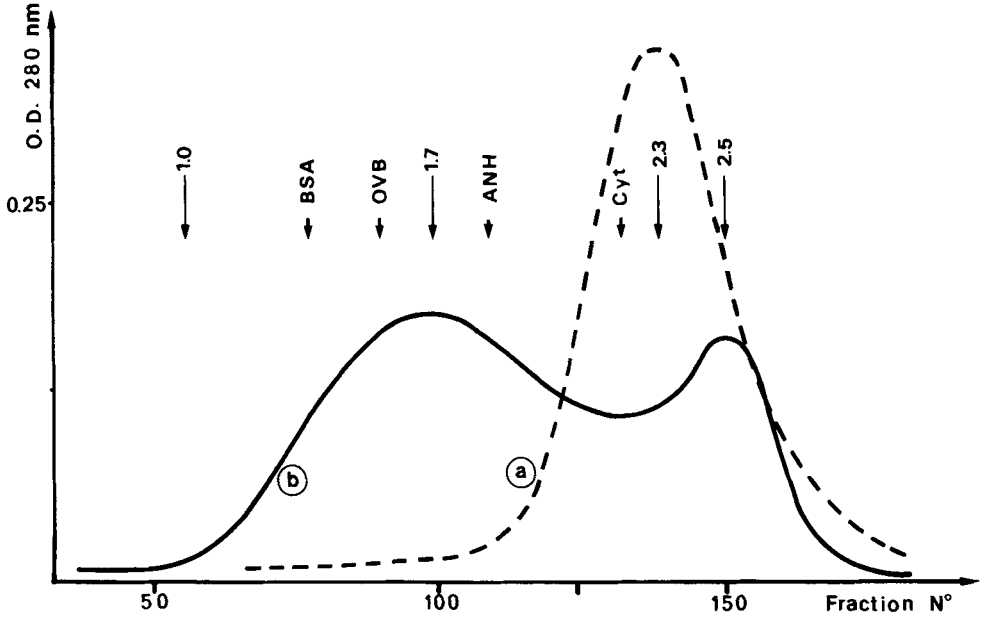

Fig. 6. Polymerization activity of laccase toward a low-mw TGL corresponding to fraction F4 of Fig. 1: (**a**) An amount corresponding to 11 U of $A_{280\ nm}$ (1.44 mg TGL) of this TGL was left unreacted and loaded onto a Sephadex G-100 column; (**b**) an amount of 51 U of $A_{280\ nm}$ (6.7 mg TGL) was reacted with 12,200 U of laccase in a total vol of 4 mL and an aliquot representing 11.6 U of $A_{280\ nm}$ (1.03 mg laccase-oxidized TGL; *see* comment in Fig. 5 b) of this reaction medium was loaded onto the Sephadex G-100 column.

carrying out two successive reactions in the following conditions (*see* Fig. 7 below):

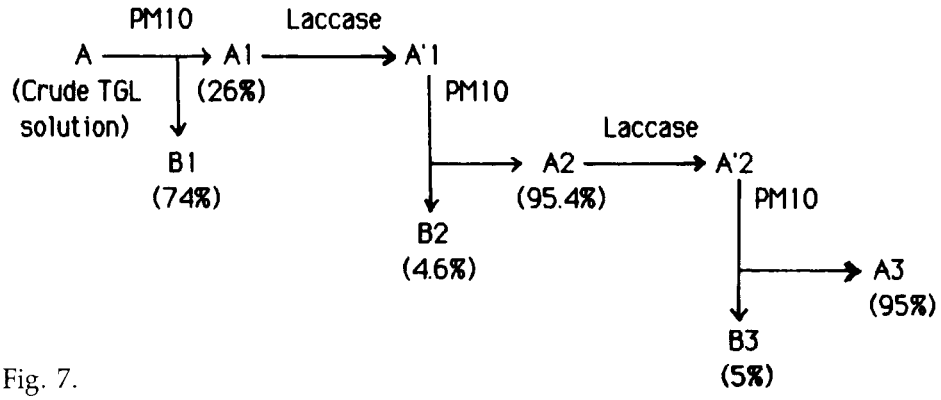

Fig. 7.

The purpose of this procedure was to eliminate the degradation products of the first incubation step before initiating the second incubation step. Reaction media were fractionated by ultrafiltration on Amicon PM10 membrane. Fractions A1, A2, and A3 corresponded to high-mw polymers (more than 10 kdaltons in molecular mass) and B1, B2, and B3 contained depolymerization products (less than 10 kdalton in molecular mass). Finally, the numbers between brackets are the percentages of total material recovered in each fraction [A1 (26%) means that fraction A1 contains 26% of the total material recovered after ultrafiltration; Σ A1 + B1 = 100%].

The results strongly favor the hypothesis of an equilibrium between condensation and depolymerization reactions. The latter should continue if the equilbrium is artificially shifted, i.e., by removing degradation products from the reaction medium.

The spectral analysis of these fractions indicated very significant data. The $\Delta\epsilon i$-curves of the fraction A, A1, and B1 were typical of nonoxidized TGL (Fig. 4A and C, curve a), those of fractions A2, A3, and B3 were typical of oxidized TGL (Fig. 4B and C, curve b) and the $\Delta\epsilon i$-spectrum of B2 presented a peak at 335 nm (Fig. 4C, curve c).

Only fraction B2 contained the component with this specific spectral characteristic. This suggests that the TGL molecule contains in its structure chemical bonds that were easily broken by the laccase. Another possibility was that the TGL solution contained several molecular species with different structures, some of which were preferential substrate for the enzyme. Subsequent experiments involving the purification of fraction B2 on Sephadex G-25 led to the separation of two peaks. The $\Delta\epsilon i$-spectrum of the dominant peak exhibited a maximum at 335 nm, without shoulders at 365 and 255 nm, showing that the component was probably isolated with a satisfactory degree of purity. The next step in our work will be the characterization of this compound.

DISCUSSION

The mechanisms of lignin degradation are far from being completely understood. Nevertheless, it is generally assumed that the macromolecule is depolymerized, via an oxidative process. In this respect, laccase has long been considered as governing the lignin-degrading capacity of fungi. However, as reported in the Introduction section, the function of this enzyme remains unclear.

The results of the experiments that we performed using the purified laccase L1 of *R lignosus* have shown that this enzyme is able to partially depolymerize the macromolecule. A condensation activity has also been unequivocally evidenced. In vitro, these two reactions most probably undergo a regulation mechanism based on an equilibrium between the reaction products.

These results agree with other published data (*16,43*) on the laccase effect on either milled wood lignin or lignosulfonate. They also agree with the hypothesis of Kirk et al. (*19*), who predicted the two types of laccase action as a result of the radical formation of phenylpropan units (those showing at least one free phenolic hydroxyl group), leading to either condensation products or to the cleavage of the aryl—alkyl bonds. In spruce lignin, only 41% of the units may undergo such an oxidative cleavage (*19*). Total lignin breakdown needs, therefore, the occurrence of either other mechanisms or enzymes.

Among the latter hypotheses of note is the report by Ander and Eriksson, who evidenced the basic role of *Phanerochaete chrysosporium* peroxidase in lignin degradation (*27*). The function of this type of enzyme was also investigated by Brunow and Wallin (*44*), who showed that it essentially polymerized the lignosulfonate, and Lobarzewski et al. (*45,46*), who reported its ability to both condense and degrade this macromolecule.

Recently, Tien and Kirk (*47,48*) purified, from *P. chrysosporium* culture filtrates, a ligninase they called H_2O_2-requiring oxygenase. This enzyme, which produces free radicals in phenylpropan units, even in the absence of any free phenolic hydroxyl group, was shown both to cleave alkyl—aryl bonds of dimeric model compounds and to degrade the lignin polymer. It was also isolated by Glenn et al. (*49*) and Gold et al. (*50*) and characterized as a peroxidase by Shoemaker et al. (*51*) and Harvey et al. (*52*). Is this enzyme the same as the peroxidase formerly identified by Ander and Eriksson, or does it act in concert (or in synergy) with the latter?

More generally, our findings, and those recently reported, reveal that several oxidative enzymes may be involved in lignin degradation. Laccase is one such enzyme. Nevertheless, further studies are needed for understanding the regulation mechanisms governing the in vivo intervention of the different enzymes in lignin degradation.

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