Laccases of *Rigidoporus lignosus and Phellinus noxius*

I. Purification and Some Physicochemical Properties

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ABSTRACT

Three phenol oxidases, of which two are excreted by *Rigidoporus lignosus* and one by *Phellinus noxius,* have been isolated and purified from culture filtrates. Based on their substrate specificities and spectral characteristics, these enzymes are *p*-diphenol:oxygen oxidoreductases (laccases; EC 1.10.3.2). A number of their physicochemical properties have been determined. The fact that the two parasites excrete laccases indicates that they belong to the group of lignin degrading (white rot) fungi. Laccase L1 from *R. lignosus* was purified to homogeneity.

Index Entries: *p*-Diphenol oxidase; laccase; enzyme purification; white rot fungi; *Rigidoporus lignosus; Phellinus noxius; Hevea brasiliensis.*

INTRODUCTION

In a previous study on host–parasite interactions between *Hevea brasiliensis* and *Rigidoporus lignosus*, we have shown that the fungus excretes two phenol oxidases that were assumed to be laccases (1,2). More recently, investigations were carried out on another rubber tree parasite,

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Phellinus noxius, considered a brown rot fungus, which also secretes phenol oxidases (3). The aim of the first part of this study was to verify whether the phenol oxidases excreted by both fungi are indeed laccases and to purify them in order to determine and compare a number of their physicochemical characteristics. These parameters were also compared to enzymes of the same type synthesized by trees or fungi [*see* refs. (4) and (5) for review]. Finally, the ultimate aim was to purify to homogeneity, laccase L1, (the most active one) and to use it (part II of the study) for determining is potential role in lignin degradation.

MATERIALS AND METHODS

Fungal Culture

Rigidoporus lignosus and *P. noxius* were grown in 250-mL Erlenmeyer flasks containing 10 g of fresh rubber tree sawdust and 100 mL of water. The media were sterilized for 45 min at 120°C. After cooling, they were inoculated with a mycelial implant taken from a preculture in 2% malt extract and 1% agar. After incubating for 3 wk at 28°C, the cultures were filtrated through a porosity 1 sintered-glass filter, and the filtrates containing the enzyme activities were collected.

Purification Method

A small quantity of DEAE-cellulose (Whatman DE52) was directly added to the culture filtrate, previously centrifuged at 20,000g for 15 min. After 30 min with gentle stirring, the ion exchanger was recovered by filtration through a sintered-glass filter. This process was repeated until all the laccase activity was adsorbed. The DEAE-cellulose cakes were pooled, and the enzymes were eluted with a sodium phosphate buffer (0.0125M at pH 6) containing sodium chloride (0.5M). The elution was repeated three times in order to recover most of the initial laccase activity. This step allows concentration of the enzyme preparation.

The eluate was centrifuged and concentrated in an Amicon cell using a PM10 ultrafilter (cut-off at 10 kdaltons). The concentrate was rediluted 50-fold by adding 0.025M Tris-HCl buffer at pH 8.4 and concentrated again. This operation was repeated three times; it replaces conventional concentrating dialysis and allows for buffer changing.

DEAE-Cellulose Column Chromatography

The enzyme solution was loaded on a DEAE-cellulose (Whatman DE52) column ($d = 2$ cm; $h = 18$ cm) equilibrated with a 0.025M Tris-HCl buffer at pH 8.4. Elution was achieved by a linear gradient of sodium chloride (0–0.35M). This step separates laccases L1 and L2, contained in the culture filtrate of *R. lignosus,* from each other.

Hydroxylapatite Chromatography

A column (d = 1.5 cm, h = 8 cm) was packed with a mixture of hydroxylapatite–cellulose (Whatman F1) (3/1, w/w) previously equilibrated in a 0.01M sodium phosphate buffer at pH 6. Proteins were eluted stepwise with pH 6 phosphate buffers at the following concentrations: 0.01, 0.03, and 0.05M. This chromatography step was used only to purify the *R. lignosus* laccases.

Sephadex Gel Filtration

Sephadex gel filtration was performed on columns (d = 1.8 cm, h = 100 cm) using Sephadex G-75 or G-100 gels previously equilibrated with a 0.05M sodium phosphate buffer at pH 6. This step was used either for enzyme purification or for molecular weight (MW) determination (6). In the latter case, the columns were calibrated with standard proteins: bovine serum albumin (BSA), 68,000; ovalbumin (OVB), 45,000; horse radish peroxidase (Pox), 49,000; carbonic anhydrase (ANH), 31,000; and cytochrome C (Cyt c), 12,500.

Isoelectric Point (pI) Determination

An LKB 8100 column was used for electrofocusing. It contained 4 mL of appropriate ampholines, leading to a 2.5–5 pH gradient that was stabilized by a linear 5–45% sucrose gradient. Each experiment lasted 3 d at 4°C and under 400 V. Fractions of 1.2 mL were collected; the pH (at room temperature) and laccase activity of each were measured.

Enzyme Purification Control

Enzyme purification control was carried out on starch gel (7,8) or polyacrylamide gel electrophoresis (7.5% acrylamide in a 0.2M Tris-HCl buffer at pH 8.5) under denaturing (9) or nondenaturing (10) conditions. The laccase activity was detected by immersing the gel in the following reaction mixture: 0.2% guaiacol and 0.03% 3-amino-9-ethyl carbazole in a 0.05M sodium acetate buffer at pH 5. Proteins were stained with a solution of 0.1% Coomassie blue in 5% acetic acid. The molecular weight of enzymes under denaturing conditions was achieved with reference to standard proteins: lysozyme, 14,400; sojabean trypsine inhibitor (STI), 21,000; alcohol dehydrogenase (ADH), 35,000; and 3-phospho-glyceraldehyde kinase (3PGK), 47,000.

Laccase Assay

Laccase activity was usually determined in a reaction medium containing 0.2% guaiacol in a 0.05M sodium phosphate buffer at pH 6; the final vol was 5 mL, and the incubation time varied between 1 and 10 min at room temperature. The activity was expressed as $A_{420\ nm} \times$ 1000/mL of

TABLE 1
Substrate Specificity of the L1, L2, and LN Laccases[a]

Substrate	Concentration, μmol/mL	Relative activity		
		L1	L2	LN
Hydroquinone	1.8	100	100	100
Guaicol	1.8	80	87	105
Syringaldazine[b]	0.12	12.5	5.3	12
Tyrosine	1.8	0	0	0
p-Hydroxybenzaldehyde	1.8	12	5	14
p-Hydroxybenzoic acid	1.8	22	0	14
o-Cumaric acid	1.8	45	28	17
m-Cumaric acid	1.8	6	7	7
p-Cumaric acid	1.8	80	56	50
Cafeic acid	1.8	190	228	374
Ferulic acid	1.8	190	244	309
Coniferylic acid	1.8	165	215	310
Sinapinic acid	1.7	3600	280	400
Thioglycolic lignin	(0.2%)	148	109	171

[a]Reaction media: total vol, 3 mL; each substrate is dissolved at the indicated concentration in a 0.05M sodium acetate buffer at pH 5.0 for the LN laccase test (2400 units/assay) or a 0.05M sodium phosphate at pH 6.0 for the laccases L1 (7350 U/assay) and L2 (1070 U/assay) test.
[b]Very weakly hydrosoluble.

enzyme solution (or per mg of proteins)/min. The protein concentration was determined with the method of Warburg and Christian (11), based on the absorbance of the protein solution at 260 and 280 nm in a 1-cm light path cell.

When substrate specificity was investigated, the activity was determined by oxygen consumption (YSI 53 oxygraph). The substrates (*see* Table 1) were dissolved in 0.05M sodium phosphate buffer at pH 6. The results are expressed as relative activities in comparison to the value recorded with hydroquinone (= 100).

RESULTS

Identification of the Laccases in the Culture Filtrates

Using starch electrophoresis, it was possible to detect two laccases (L1 and L2) in the *R. lignosus* culture filtrates and one (LN) in that of *P. noxius* (Fig. 1a). In the latter case, longer incubation times revealed two additional weak bands. Subsequent work was carried out only on the major species L1, L2, and LN.
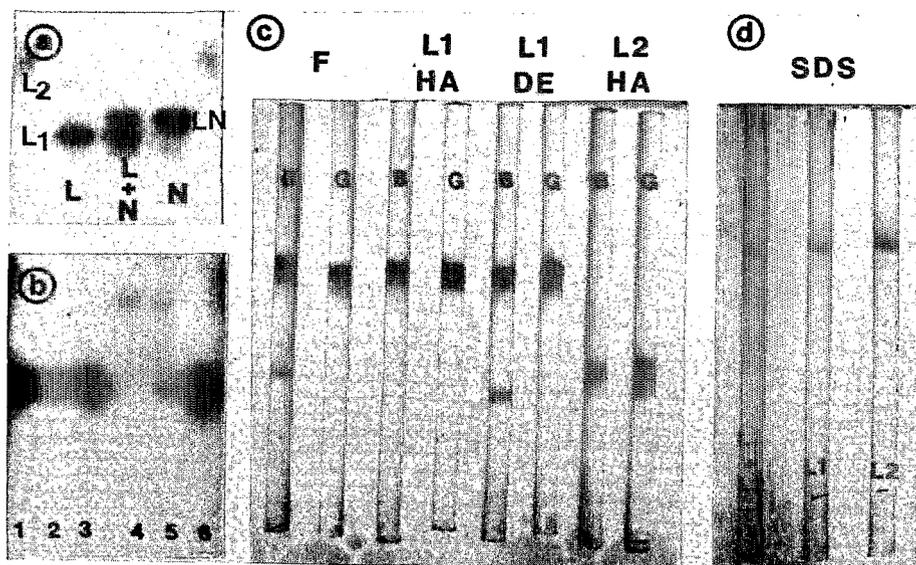
Fig. 1. (a) Laccase isozyme pattern after starch gel electrophoresis of crude culture filtrates from *R. lignosus* (L) and *P. noxius* (N); (L + N = mixture (v/v) of the two types of culture filtrates); L1 and L2 are the two laccase isoenzymes corresponding to the isoenzymes shown in Fig. 2. (b) Control of L1 and L2 at different purification steps: 1, 2, and 3, L1 fractions eluted from the hydroxylapate (HA) column; 4, L2 after the HA step; 5 and 6, respectively, L2 and L1 after the DEAE-cellulose chromatography step. (c) L1 and L2 purity control (polyacrylamide gel electrophoresis): B, Coomassie blue staining; G, laccase activity. Protein and laccase patterns: F, crude culture filtrate; L1HA and L2HA, L1 and L2 fractions after the HA chromatography step; L1DE, L1 fraction after the DEAE-cellulose chromatography step. (d) Proteinogram of the purified laccases L1 and L2 under denaturing conditions (SDS).

Purification of R. lignosus *Laccases*

The three step purification is summarized in Table 2. The crude filtrate had relatively intense brown color. Therefore, absorbance measurements (and subsequent protein concentration calculations) were not performed, since they should not have a true significance in terms of the protein content of the solution. The same was true after the first purification step (DEAE-cellulose adsorption and 0.5*M* NaCl enzyme elution), even though a considerable proportion of the pigment was already removed. The brown color was probably a result of the presence of oxidized phenolics. An important percentage of the remaining brownish color could be further removed by ultrafiltration of the solution on an Amicon PM10 membrane; it considerably improves the degree of purification. Such an apparent purification appears clearly in Table 2: after the "DE52–chromatography" step, a purification factor of 40 is noticed

TABLE 2
Purification of *R. lignosus* Laccases, L1 and L2

Purification steps	$\dfrac{OD\ 280}{OD\ 260}$	Total protein[a]	Total activity[b]	Specific activity[c]	Yield
Filtrate	—	—	6,850,000	—	100
Batch DE 52	—	—	3,575,000	—	52
DE-52 chromatography					
(a) Fractions before concentration on a PM10 ultrafilter					
L1	1.04	229.5	1,968,600	875	34
L2	0.97	296	361,300	1220	
(b) Fractions after concentration on a PM10 ultrafilter					
L1	1.27	60.5	1,892,200	31,270	33.4
L2	1.05	42.9	394,000	9200	
Hydroxylapatite after concentration on a PM10 ultrafilter					
L1	1.4	35.2	1,472,500	41,830	22
L2	1.12	5.0	37,500	9000	

[a]In mg.
[b]In units.
[c]Units/mg protein.

for the laccase L1, simply by concentrating and washing the L1 fraction on the ultrafilter.

The DEAE-cellulose column chromatography step (Fig. 2) permits the separation between laccases L1 and L2. Nevertheless, fraction L2 was still contaminated by laccase L1, as shown on the electrophoresis pattern (Fig. 1b). This contamination was eliminated by hydroxylapatite chromatography of the L2 fraction.

The hydroxylapatite column chromatography was also used for further purification of the laccase L1. This enzyme was not adsorbed on hydroxylapatite. The L1 active fractions collected in the column effluent no longer contained the protein contaminant present at the "DE-52 chromatography" step (Fig. 1c: compare L1HA and L1DE.)

Figure 1c shows the results of some of these purification steps with parallel representation of the proteinograms (Coomassie blue staining) and zymograms (laccases revealed by their catalytic activities). Finally, Fig. 1d shows protein homogeneity after polyacrylamide gel electrophoresis under denaturing conditions.

Purification of *P.* noxius *Laccase*

The enzyme *P. noxius* laccase was purified to a lesser extent, our aim being limited to obtaining a laccase solution, concentrated enough and free of the brown pigment, enabling physicochemical determinations.

The initial purification steps, DEAE-cellulose adsorption, elution with 0.5*M* sodium chloride, and concentration on an Amicon PM10
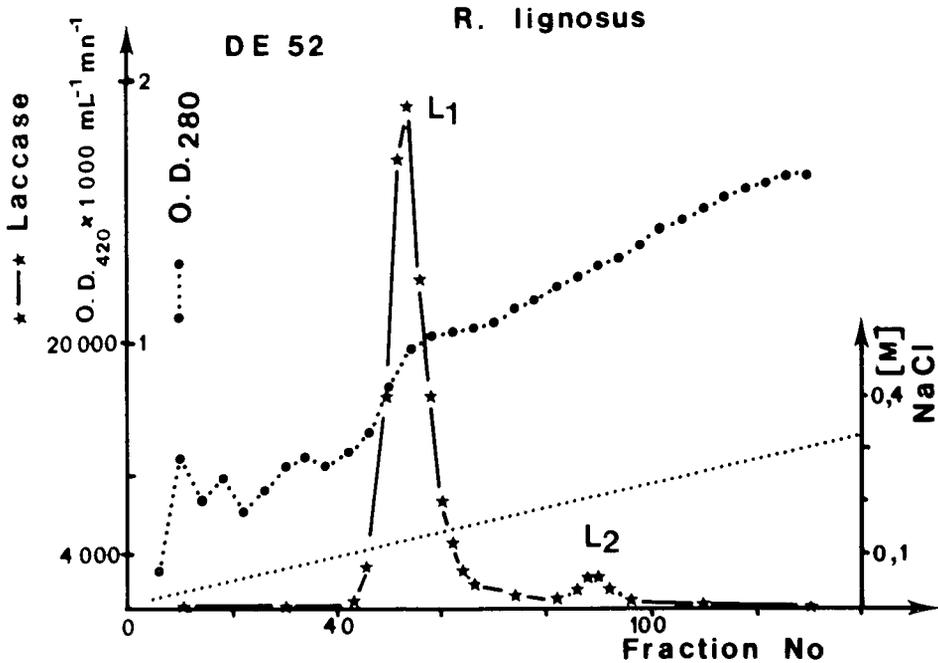
Fig. 2. Elution profile of the proteins (as OD_{280nm}) and laccases L1 and L2 on DEAE-cellulose. NaCl gradient: 0–0.35M in a Tris-HCl 0.025M, pH 8.4 buffer; total vol, 700 mL; fraction vol, 5 mL; elution speed, 60 mL/h.

membrane, were the same as described above. The resulting solution was very rich in glycosidase, polyosidase, and peroxidase activities. In order to separate them from laccase, the solution was chromatographed on a DEAE-cellulose (DE-52) column (Fig. 3). Laccase-enriched fractions still contained appreciable α-galactosidase an peroxidase activities. These contaminants were removed by Sephadex G-100 gel filtration (Fig. 4). The most laccase-active fractions were pooled and concentrated; the resulting solution exhibited a typical blue color. Weak residual peroxidase activity nevertheless persisted, as shown by the slight absorbance of the solution at 400 nm (Fig. 5B).

Physicochemical Properties

Spectral Characteristics

In addition to the protein absorbance peak at 280 nm, the spectra of laccase L1 (Fig. 5A) and LN (Fig. 5B) also showed a peak at 605 nm, which is typical for copper (Cu^{2+})-containing enzymes and responsible for the blue color of concentrated lacase solutions. Enzyme L2 presented the same spectral characteristics.
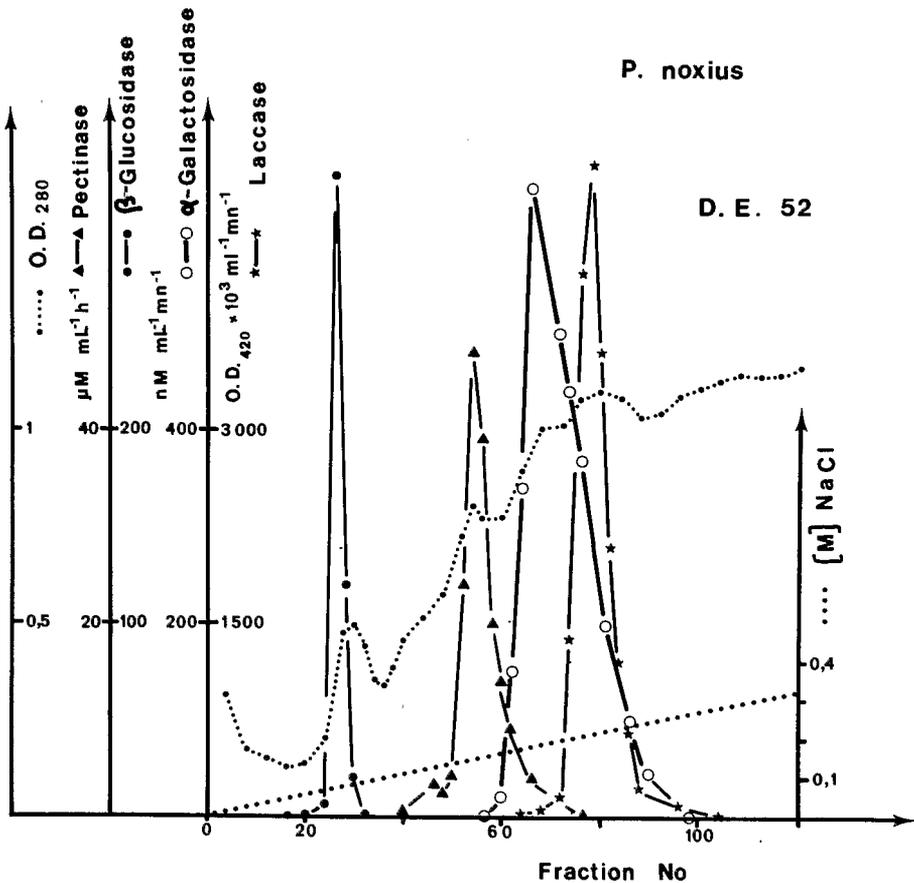
Fig. 3. Elution profile on DEAE-cellulose of total proteins and laccase, pectinase, α- and β-glucosidase activities from a *P. noxius* culture filtrate. Except for the buffer system (0.0125*M* sodium phosphate at pH6), all the other elution characteristics are as indicated in Fig. 2.

Substrate Specificity

Table 1 shows that hydrochinon and syringaldazine (*12*), specific substrates of *p*-diphenol oxidases, but not tyrosine, a typical substrate of polyphenol oxidases, are oxidized by the three phenol oxidases. This result clearly proves that these enzymes are laccases.

In addition to these three substrates, various phenolics were tested. Most were chosen because they are related to lignin metabolism. It appeared that the compounds of the cinnamic series were oxidized more rapidly than the corresponding phenolics of the benzoic series (*p*-cumaric acid > *p*-hydroxybenzoic acid = *p*-hydroxybenzaldehyde). As for the cinnamic series, the oxidation velocity increased in the following order: *p*-cumaric acid --> ferulic acid --> sinapinic acid. The methylation of the C3 hydroxyl group and the reduction of the carboxyl on Cγ to an alcohol had little effect on the enzyme activity (comparable efficiencies of caffeic
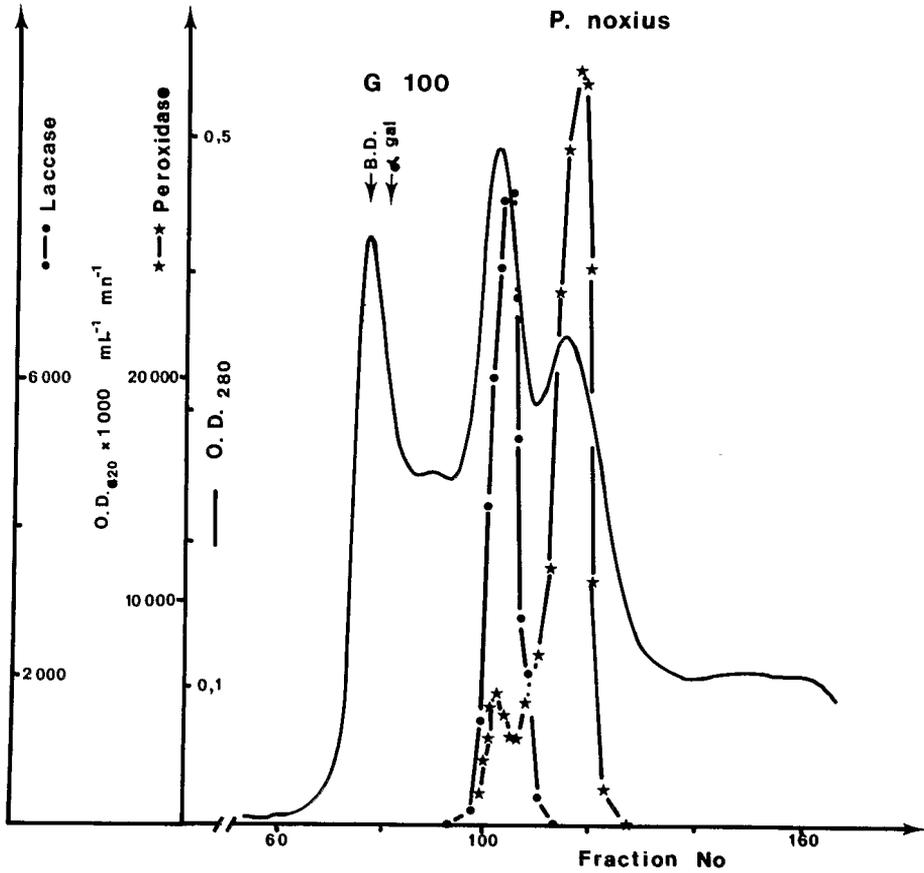
Fig. 4. Elution profile of total proteins and laccase and peroxidase activities on Sephadex G-100. The most laccase-active fractions collected after the DEAE-cellulose chromatography shown in Fig. 3 were chromatographed.
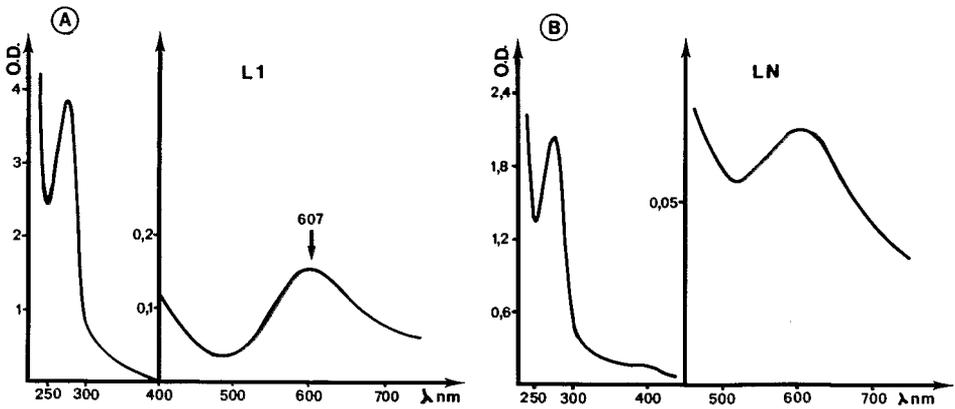
Fig. 5. Spectral characteristics of *R. lignosus* L1 (A) and *P. noxius* LN (B) laccases.

acid, ferulic acid, and coniferyl alcohol). On the contrary, as shown in other reports (*13*), the position of the free phenol hydroxyl was of fundamental importance, the para position being the most favorable and the meta the most unfavorable. The lignin that we had previously extracted from rubber tree wood by the use of thioglycolic acid (*14*) was also a substrate for the laccases.

According to a previous report (*15*), we noticed that the use of an "old" substrate, i.e., a slightly oxidized one, considerably increased the rate of the enzymatic reaction and suppressed the "acceleration" phase observed at the reaction initiation. Therefore, specific activities were always measured with freshly prepared reaction media.

The list of substrates presented in Table 1 is not limitative. Previous qualitative tests using an *R. lignosus* crude culture filtrate led us to identify about 30 phenolic substrates (*16*).

Molecular Weight Determination

The molecular weights of the nondenaturated laccases, estimated by the Sephadex gel-filtration method, were 52,000, 55,000, and 70,000, respectively, for L1, L2 (Fig. 6A), and LN (Fig. 6B).

Under denaturating conditions (SDS-polyacrylamide gel electrophoresis), the molecular weights of L1 and L2 (LN was not investigated) were 54,000 and 55,000, respectively (separate runs). Nevertheless coelectrophoresis of these two enzymes revealed the presence of one protein band only (Fig. 1d).

These results indicate that laccases L1 and L2 are single polypeptide-chain proteins having very close (if not identical) molecular weights.
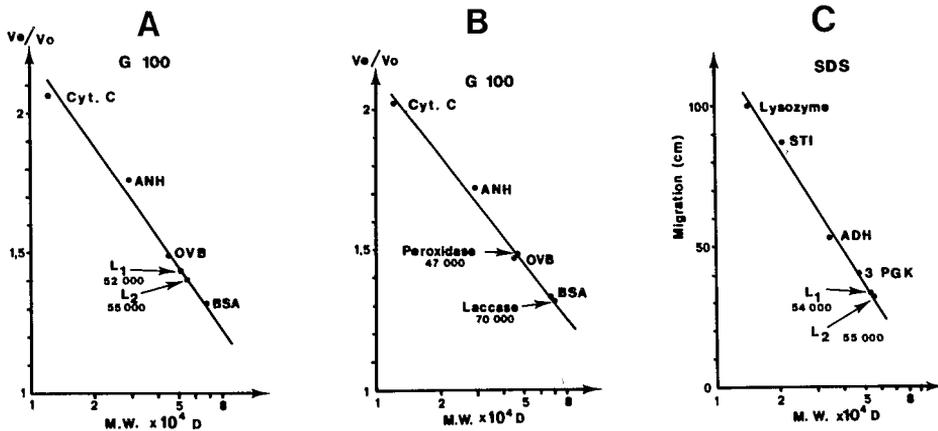
Fig. 6. Molecular weight estimation of *R. lignosus* L1 and L2 laccases (A) and the *P. noxius* enzyme (B) under nondenaturating conditions. These determinations were performed with the Sephadex gel filtration method. (C) MW determination (SDS-polyacrylamide gel electrophoresis) of L1 and L2 under denaturating conditions. Previous to the electrophoresis, the proteins were denaturated by incubation for 10 min at 100°C in a Tris-HCl 0.2M, pH 8.5, buffer, containing 1% SDS.

Optimal pH and Isoelectric Point Determination

The catalytic activities of laccases L1, L2, and LN, using guaiacol as substrate [the optimal pH may vary as a function of the substrate (*17*)] were optimal at pH 5.6, 6.0, and 4.0, respectively.

The p*I* of laccase L1 was 3.83 and that of L2, 3.32. These differences in their p*I* may explain the differential electrophoretic migration and chromatographic behavior on DEAE-cellulose of these two enzymes having similar molecular weights.

DISCUSSION–CONCLUSION

The aim of the present investigation was to purify the laccases excreted by *R. lignosus* and *P. noxius* and to determine some of their physicochemical characteristics (Table 3). The methods used were conventional for protein purification. The behavior of laccases L1 and L2 on DEAE-cellulose was similar to laccases B and A purified by Mosbach (*18*) and by Fahreus and Reinhamar (*19*) from *Polyporus versicolor*.

Laccases L1 and L2 were usually electrophoretically homogeneous after the last purification step. In some cases, however, after polyacrylamide gel electrophoresis of the L1 laccase, we could distinguish, very close to the major band, two weak satellite bands exhibiting catalytic activity. They were not detected under denaturating conditions, suggesting that they have the same molecular weight as the major form. These species may result from a partial, but minor, degradation of the original L1 protein. The presence of relative active proteases and some glycosidase activities in *R. lignosus* culture filtrates is consistent with this hypothesis (laccases are known to be glycoproteins).

The existence of multiple forms of laccases has been reported by Evans and Palmer (*20*), who have separated *Coriolus versicolor* laccase B into five fractions (p*I* comprised between 4.5 and 6.5). The laccase A was

TABLE 3
Some Physicochemical Properties of L1, L2, and LN Laccases

	L1	L2	LN
Protein MW			
nondenaturated	52,000	55,000	70,000
denaturated	54,000	55,000	—
Optimal pH (guaiacol)	5.6	6.0	4.6
Isoelectric point	3.83	3.32	—
KCN (0.1 m*M*) inhibition (%)	66	73	87
OD280/OD605	24.6	—	29.0
Specific activity (at pH 6; guaiacol as substrate)	41,800	9,000	5,300

also separated into two molecular species. When the homogeneity of the purified laccases L1 and L2 was controlled by electrofocusing in polyacrylamide gels, we could also observe multiple protein bands exhibiting catalytic activity and having very different p*I* values (comprised between 3 and 6). Additional experiments showed that this separation into multiple forms no longer occurred when electrofocusing in polyacrylamide gel was preceded by a "prerun" for 30 min at 150 V. This indicates that the molecular structure of laccases L1 and L2 may be relatively fragile, or that these proteins may interact with a chemical contained by the gel (perhaps the persulfate). Therefore, the electrophoreses in polyacrylamide gels were always preceded by a blank-run, to avoid the occurrence of artifacts.

Enzymes L1, L2, and LN are really laccases, according to their spectral characteristics (absorbance peak at 605 nm, blue color of purified laccase solutions) and their substrate specificity. As reported (4,21,22), this specificity is very broad, including monophenols, *o-*, *m-*, and *p*-diphenols, a variety of substituted phenolics, thioglycolic lignin, and so on.

The molecular weights are in the range frequently reported for other fungal laccases (5); only enzyme 1 of *Podospora anserina* (MW = 390,000) seems to be an exception (23,24). The pH optima of the three laccases and their p*I*s are standard for fungal laccases and differ from those of plant enzymes, such as that of *Rhus vernicifera* (25).

More generally, it should be noted that the laccases have been extensively investigated (4,5). In addition to developing purification methods and determining physicochemical properties, a number of reports deal with the stimulation of enzyme excretion by various phenolic compounds (26–30), the different forms of copper bound to the protein, and their role in enzymatic catalysis (31–34), and the value of laccases for chemotaxonomy (35–36).

Nevertheless, for pathologists and authors studying wood degradation, the excretion of laccases by fungi has another significance. In fact, since the report of Bavendamm (37), it was shown that the lignin degrading white rot fungi excrete laccases, whereas the brown rot fungi that do not degrade lignin do not excrete those enzymes. As a consequence, *P. noxius*, which was formerly considered a brown rot fungus, must be reclassified among the white rot fungi group. The finding of Bavendamm also raised the question of whether laccases are responsible for lignin breakdown. Therefore, using the purified laccase L1, further experiments were performed (part II of this study) in order to determine the effect of this enzyme on thioglycolic lignin.

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