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ISOLATION, PURIFICATION, AND STUDY OF SOME PROPERTIES OF THE EXTRACELLULAR LIGNIN PEROXIDASES OF THE WOOD-DESTROYING FUNGUS *Pleurotus ostreatus*

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Two bands with lignin peroxidase activity have been detected by isoelectric focusing in a total enzyme preparation obtained from a 15-day filtrate of the culture liquid of the fungus Pleurotus ostreatus *by fractionation with ammonium sulfate. Two homogeneous forms of the enzymes — LGP-I and LGP-II — have been obtained by gel filtration on Sephadex G-100, ion-exchange chromatography on DEAE-Toyopearl 650 M gel, and rechromatography on Sephadex G-75, and also by electrophoresis in PAAG. The specific activities of the purified lignin peroxidases LGP-I and LGP-II amounted to 36.5 and 54.3 units/mg, their degrees of purification being 8.7 and 12.9, respectively. The molecular masses of LGP-I and LGP-II, determined by electrophoresis in PAAG in the presence of Na-DS and by gel filtration on TSK HW-65 gel were 42-44 and 61-63 kDa. The isoelectric points of LGP-I and LGP-II were 3.4 and 4.1, their pH optima 2.7 and 3.4, and the temperatures of their optimum enzymatic action 28 and 34°C, respectively. The isoenzymes differed from one another substantially with respect to pH stability and resistance to heat. The values of K_M determined from the rates of hydrolysis of the substrate by the enzymes in the presence of H_2O_2 at pH 3.7 were 0.09 mM for LGP-I and 0.07 mM for LGP-II. The values of K_M with respect to veratryl alcohol, determined by the Lineweaver–Burk method, were 0.117 mM for LGP-II and 0.132 mM for LGP-II.*

In recent years, investigations of the enzyme systems of basidial fungi actively participating in the delignification of lignin-containing raw material have been widely developed throughout the world. Analysis of the literature shows that the main components of the ligninolytic complex are considered to be lignin peroxidase (LGP), laccase, and Mn-dependent peroxidase [1]. The other enzymes in the complex are ascribed an auxiliary role. The question of the role and functions of each enzyme in the ligninolytic complex and of the nature of the actions of the individual enzymes of this complex remains under discussion.

The capacity of the basidial fungus *Pleurotus ostreatus* for forming ligninases on media containing various lignocellulose substrates has been reported previously [2]. Moreover, in a comparison of the ligninolytic activities of certain basidial fungi it has also been confirmed that *P. ostreatus* is the best producing agent of ligninolytic enzymes [3]. The conditions for the optimization of enzyme formation and the isolation of an active extracellular LGP enzyme preparation from a culture liquid of this fungus have also been reported [4].

The present work is a continuation of these investigations and deals with the purification and some physicochemical and biochemical properties of the LGP formed by the fungus *P. ostreatus* UzBI-I108.

To purify the LGP we used the culture liquid (CL) of the fungus obtained in the period of maximum ligninolytic activity of a culture of *P. ostreatus* grown on a 1% extract of a spent cottonseed meal pulp. As the first stage of purification, common for all the enzymes, we used precipitation of the proteins from a CL filtrate with 40% ammonium sulfate. The resulting precipitate was separated off by centrifugation and was desalted on a column of Bio-Gel P-6. The protein fractions were combined, lyophilized, and designated as preparation 1. The methods of obtaining this preparation and its total ligninolytic activity have been described in detail in [4].

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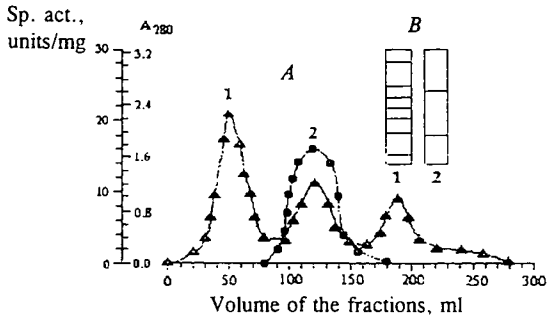


Fig. 1. Gel filtration of the LGP of *P. ostreatus* on Sephadex G-100 (*A*), and IEF activities (*2*) of the fractions in PAAG with Ampholines in the pH range of 3-10 (*B*): *A*: *1*) protein; *2*) specific LGP activity, units/mg; *B*: *1*) Coomassie-stained protein; 2) LGP activity.

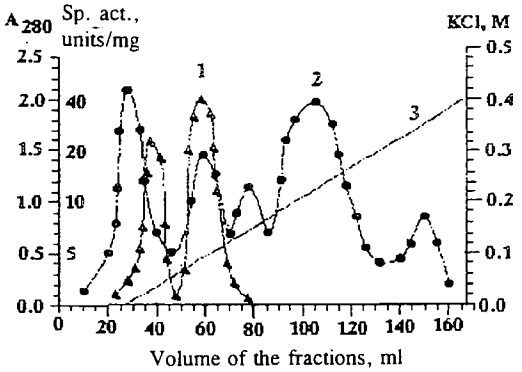


Fig. 2. Ion-exchange chromatography of the *P. ostreatus* LGP on DEAE-Toyopearl 650 M gel: *1*) specific LGP activity; *2*) protein, A_{280}; *3*) KCl concentration, M.

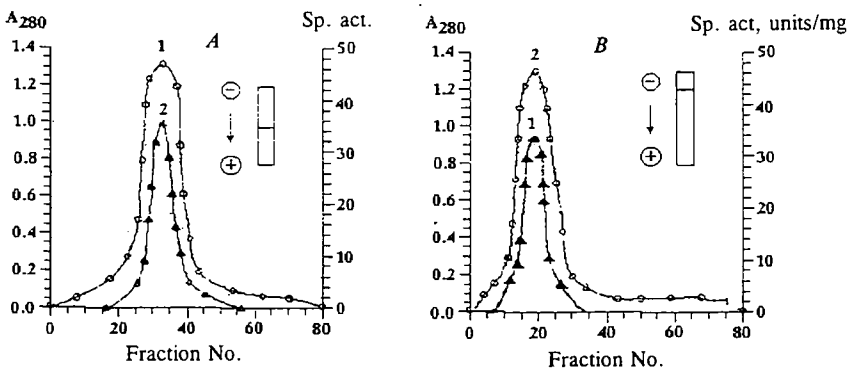


Fig. 3. Gel filtration of LGP-I (*A*) and LGP-II (*B*) on Sephadex G-75: *1*) protein, A_{280}; *2*) LGP, specific activity, units/mg.

Preparation 1 was subjected to gel filtration on a column filled with Sephadex G-100 (Fig. 1). Protein was eluted from the column as three fractions (fraction 1, elution volume 78 ml; fraction 2, 145 ml; fraction 3, 205 ml), LGP activity, expressed as a single broad peak, being detected in fraction 2. Electrophoresis of this active fraction revealed seven protein bands, only two of which possessed LGP activity.

TABLE 1. Results of the Purification of the Extracellular LGP of *P. ostreatus*

Stage of purification	Total protein, mg	Total activity	Specific activity	Degree of purification	Yield, % act.
Filtrate of the culture liquid	12000	50400	4.2	1.0	100
Precipitation with ammonium sulfate, 660 g/liter	5253	35195	6.7	1.6	100
Gel filtration on Sephadex G-100	2539	36816	14.5	3.45	73.1
Ion-exchange chromatography on DEAE-Toyopearl 650 M					
fraction I	514.7	11684	22.7	5.40	23.2
fraction II	296.4	12241	41.3	9.83	24.3
Rechromatography on Sephadex G-75					
LGP-I	233.2	8512	36.5	8.7	16.9
LGP-II	122.5	6652	54.3	12.9	13.2

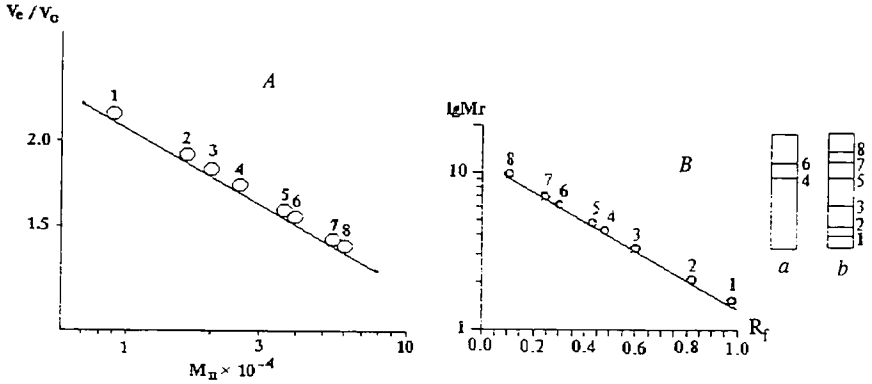


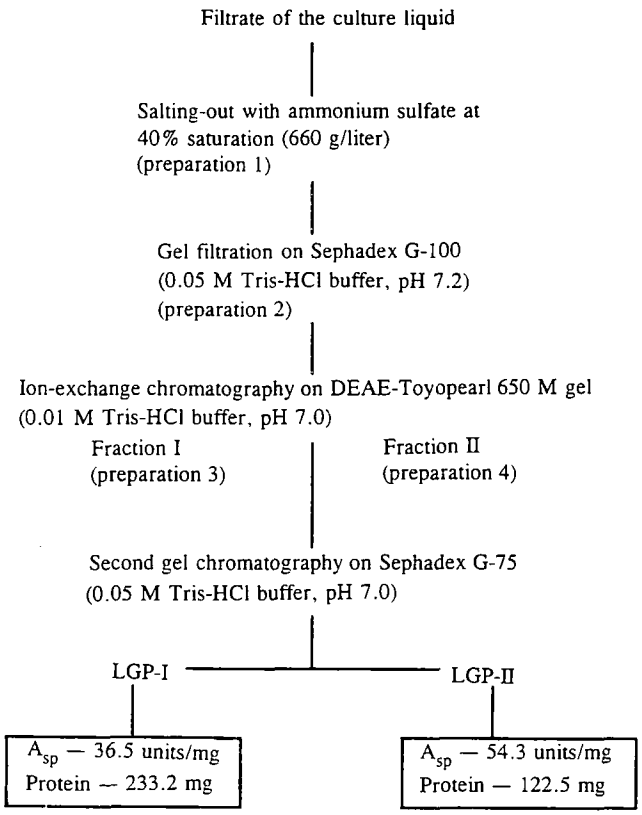
Fig. 4. Determination of the molecular masses of LGP-I and LGP-II by gel filtration on TSK HW-65 (*A*) gel and from their mobilities on electrophoresis in PAAG (*B*). *A*: *1*) Cytochrome (12,300); *2*) soybean trypsin inhibitor (20,000); *3*) trypsin (24,000); *4*) carboanhydrase (30,000); *5*) LPG-I (44,000); *6*) ovalbumin (45,000); *7*) LGP-II (63,000); *8*) BSA (67,000). *B*: *1*) Cytochrome; *2*) trypsin; *3*) carboanhydrase; *4*) LGP-I (42,000); *5*) ovalbumin; *6*) LGP-II (62,000); *7*) BSA; *8*) gelatin (95,000). Electrophoregrams of proteins: *a*) LGP and *b*) LGP-II; *b*) markers.

At this stage of purification it was possible to eliminate about 60-70% of accompanying substances from the enzyme. The fractions exhibiting LGP activity were concentrated by ultrafiltration through a UM-2 membrane and were subjected to ion-exchange chromatography (IEC). The unadsorbed proteins were first eluted with 0.01 M Tris-HCl buffer, pH 7.0, and then the ionic strength of the buffer was raised linearly to 0.05 M. The adsorbed proteins were eluted with the same buffer containing 0.02 M NaCl.

This successfully eliminated ballast substances from the enzyme. Fractions exhibiting LGP activity were eluted at a KCl concentration of 0.4 M in 0.05 M Tris-HCl buffer, pH 7.0. Enzyme proteins exhibiting LGP activity were eluted as two peaks (Fig. 2); i.e., by IEC it was possible to separate from the accompanying proteins two fractions (I and II) with high LGP activities.

The further purification of the LGP was conducted by chromatofocusing fractions I and II on a column of Sepahadex G-75. The proteins of fractions I (Fig. 3*A*) and II (Fig. 3*B*) were eluted as single symmetrical peaks. The active fractions, containing LGP-I and LGP-II, were combined and lyophilized.

We have developed a scheme for obtaining purified LGPs from the CL of the fungus *P. ostreatus* that includes fractionation with ammonium sulfate, gel filtration through Sephadex G-100, ion-exchange chromatography on the gel DEAE-Toyopearl 650 M, and rechromatography on Sephadex G-75.



Scheme 1. Purification of the extracellular lignin peroxidases of the fungus *P. ostreata*

The specific activities of the purified enzymes LGP-I and LGP-II were 36.5 and 54.3 units/mg, respectively. The degree of purification of LGP-I was 8.7-fold and that of LGP-II 12.9-fold. Table 1 gives information on the purification of the LGP of the fungus *P. ostreatus*. It can be seen from Table 1 and Fig. 2 that ion-exchange chromatography on DEAE-Toyopearl 650 M permits a good separation of these fractions. On subsequent rechromatography of both fractions, homogeneous enzyme preparations were obtained.

The specific activities of the *P. ostreatus* isoenzymes LGP-I and LGP-II, 36.5 and 54.3 units/mg, and their isoelectric points, 3.4 and 4.1, correspond to literature figures for ligninases from other sources [5-8].

Each of the isolated isoenzymes of *P. ostreatus*, LGP-I and LGP-II appeared in the form of a single band on electrophoresis under denaturing conditions.

The molecular masses of the lignin peroxidases were determined by gel filtration on a column of TSK HW-65 gel as 44 and 63 kDa, respectively (Fig. 4*A*). The rather high molecular mass of LGP-II (63 kDa) was unexpected, as the molecular masses of ligninase isoenzymes usually amount to 37-45 kDa [9]. The same results (42 and 61 kDa, respectively) were obtained by an independent method — electrophoresis in PAAG in the presence of Na-SD (Fig. 4*B*). Thus, from the culture liquid of the fungus *P. ostreatus* we have obtained two forms of LGP, differing in specific activity, isoelectric point, and molecular mass.

The ligninolytic complex of *Ph. chrysosporium*, which includes a LGP and a Mn-dependent peroxidase, has been studied in the most detail [10-16]. It has recently been established that other white rot fungi — *Coriolus versicolor* [17] and *Phlebia radiata* [18] — also produce peroxidases similar in their properties and the mechanism of their action to the LGP from the fungus *Ph. chrysosporium*. This shows the existence of general laws of the biodegradation of lignin for all white rot fungi, and, in particular, for the fungus *P. ostreatus*.

A study of the pH dependence of the LGPs of the fungus *P. ostreatus* showed that the enzymes oxidized the substrate to the maximum extent in the acid pH region, the pH values for optimum action being 2.8 and 3.6 for LGP-I and LGP-II, respectively (Fig. 5*A*). At higher pH values the activity of the LGPs diminished. This is probably connected with the depro-

TABLE 2. Some Properties of the Purified LGPs of the Fungus *P. ostreatus*

Index	LGP-I	LGP-II
Molecular mass, kDa		
a) gel filtration on TSK HW-65 gel	44	63
b) disk electrophorsis in 15% PAAG with Na-DS	42	61
Isoelectric point	3.4	4.1
pH optimum	2.7	3.4
Temperature optimum	28°	34°
pH stability	3.5	4.7
Temperature stability	38°	42°
K_M (at pH 3.7, mM substrate)	0.09	0.07
K_M (Lineweaver-Burk,mM)	0.132	0.117

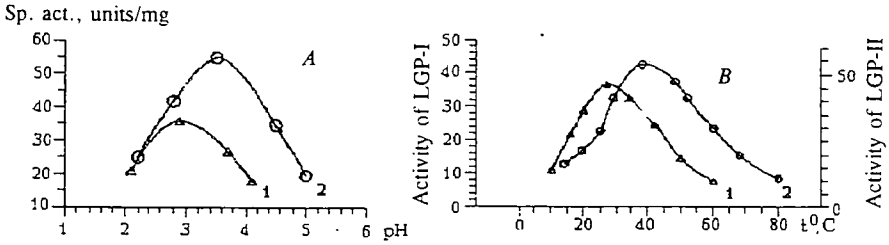


Fig. 5. Activities (*A*) of LGP-I (*1*) and of LGP-II (*2*) as functions of the pH of the reaction mixture, and temperature optima (*B*) of LGP-I (*1*) and LGP-II (*2*).

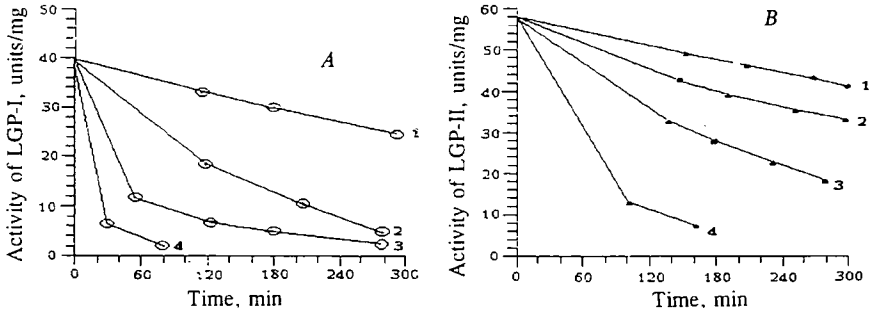


Fig. 6. Curves of the heat inactivation of LGP-I (*A*) and LGP-II (*B*): *1*) 40°C; *2*) 50°C; *3*) 60°C; *4*) 70°C.

tonation of the active groups of the enzymes that are necessary for catalysis, since at pH > 4 ligninase activity is suppressed, and at pH > 5 no LGP activity can be detected.

The pH optimum of 2.7 obtained for LGP-I agrees with literature information [6, 7, 9]. Such values of the pH optima are probably explained by the fact that the formation of the cation radicals that participate in the reaction takes place predominantly in an acid medium.

The isoenzymes also differed in the temperature optima of their action. The temperature optima of LGP-I and LGP-II were, respectively, 27 and 38°C (Fig. 5*B*). LGP-II showed its activity to the maximum extent at high temperatures (38-40°C), and with a rise in the temperature the activity of the enzyme in the reaction mixture gradually decreased. Complete loss of activity by the enzymes took place at 60-65°C for LGP-I and at 85-90°C for LGP-II.

The study of the pH and temperature stabilities of the two forms of the enzyme showed that LGP-II had higher pH and temperature stabilities (Table 2). The ligninases were very unstable at high temperatures. Figure 6 gives kinetic curves of the inactivation of the LGP isoenzymes at 40, 50, 60, and 70°C. In the case of LGP-I (Fig. 6*A*) at 50°C 60% of the initial activity was retained for 2 h, and at 60°C about 40% for 1 h, while with a rise in the temperature to 70°C there was an almost complete loss of the activity of the enzyme in 30 min. The isoenzyme LGP-II (Fig. 6*B*) proved to be slightly more heat-stable: at 70°C it was stable for 1.5 h, and at 60°C it retained 50% of the activity for 3 h. At room temperature, LGP-I retained 65% of its initial activity for five days, and LGP-II retained 70% of its activity for 12 days. The activity of the unpurified preparation 1 did not change during this time.

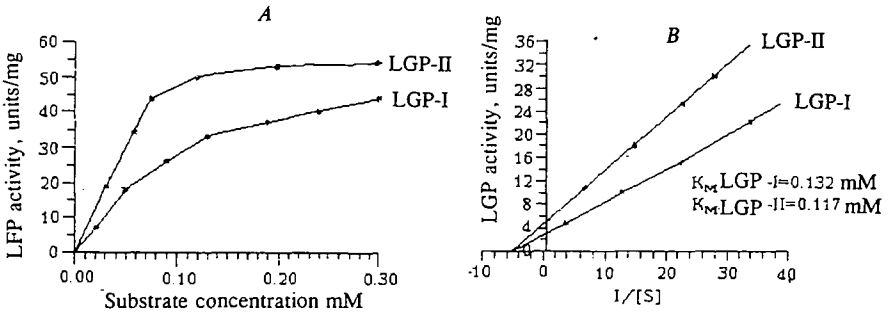


Fig. 7. Values of the Michaelis constant for LGP-I and LGP-II (*A*) and kinetics of the change in Lineweaver–Burk activity (*B*) as functions of the substrate concentration.

The values of the Michaelis constant (K_M), determined from the rate of hydrolysis of the substrate by the enzyme in the presence of H_2O_2 at pH 3.7, were 0.09 mM for LGP-I and 0.07 mM for LGP-II. The values of K_M for veratryl alcohol determined by the Lineweaver–Burk method were 0.132 and 0.117, respectively. Figure 7 gives kinetic parameters: the dependence of the initial rate of the reaction (*A*) and of the Lineweaver–Burk K_M values (*B*) on the substrate concentration for the LGPs from the fungus *P. ostreatus*. The values obtained agree with literature figures; for example, K_M for the action of *P. chrysosporium* ligninases on veratryl alcohol amounts to 0.086-0.48 mM [9, 10]

The results of a study of some properties of the purified lignin peroxidases of *P. ostreatus* are given in Table 2.

Thus, the extracellular ligninolytic complex of the fungus *P. ostreatus* UzBI-I 108 grown in a medium including an extract of spent cottonseed pulp contains two enzymes: LGP-I and LGP-II. By column chromatography on Sephadex G-100 and IEC on DEAE-Toyopearl 650 M gel and further gel chromatography on Sephadex G-75 it has been possible to obtain in the homogeneous state two forms of the enzyme: LGP-I and LGP-II differing in molecular mass (42-44 and 61-63 kDa), isoelectric point (3.4 and 4.1), pH optimum (2.7 and 3.4), temperature optimum (28 and 34°C), pH stability (3.5 and 4.7), temperature stability (38 and 42°C), K_M from the initial rate of the reaction in the presence of H_2O_2 (0.09 and 0.07 mM), and K_M for veratryl alcohol (0.117 and 0.132 mM), respectively.

EXPERIMENTAL

A local strain of the fungus *Pleurotus ostreatus* UzBI-I 108 was isolated from a poplar trunk. From slope wort-agar media the fungus was sown onto a previously sterilized nutrient medium containing 0.3% wort and 1% aqueous extract of spent cottonseed meal pulp obtained by pressing the substrate (with particle dimensions of 0.01-0.1 mm, ground in a ball mill) at high pressure in a IBFM press. Cultivation was carried out by the deep method at 28-29°C in 500-ml Erlenmeyer conical flasks each containing 200 ml of nutrient medium for 30 days on a circular shaking machine with a speed of rotation of 250 rpm.

The amounts of protein in the samples were determined by Lowry's method [21], and, during purification, spectrophotometrically at 280 nm. The LGP activities in the eluates were determined by a spectrophotometric method involving the rate of oxidation of 3,4-dimethoxybenzyl (veratryl) alcohol to veratraldehyde [22]. The absorption kinetics were measured on a Shimadzu scanning double-beam spectrophotometer (Japan) for 3 minutes. As the unit of activity we took the amount of enzyme required for the formation of 1 μmole of veratraldehyde per minute per 1 ml (1 mg) of enzyme at 20°C [sic].

A total LGP preparation was obtained by fractionation with ammonium sulfate, as described in [4].

At 40% saturation with the salt the total preparation 1 was obtained, with a specific total LGP activity of 4.2 units/ml and a protein yield of 12,000 mg. The precipitate was dissolved in distilled water and desalted on a column (5 × 50 cm) filled with Bio-Gel P-6 (Bio-Rad, USA) equilibrated with 0.03 M sodium acetate buffer, pH 5.65. Preparation 1 was deposited on a column of Sephadex G-100 (Calbiochem, USA) previously equilibrated with 0.05 M Tris-HCl buffer, pH 7.2. Elution was conducted with the same buffer at a rate of flow of 24 ml/h, the fraction volume being 3 ml. Fractions exhibiting LGP activity were combined and lyophilized and designated as preparation 2. Then preparation 2 was dissolved in the minimum amount of buffer and deposited on a column of Toyopearl 650 M gel (Toya Soda, Japan). After washing with the starting buffer, elution of the bound protein was achieved in a 0-0.4 M linear gradient of KCl at a rate of flow of 18 ml/h with a fraction volume of

0.35 ml. The active fractions I and II were combined and were concentrated on a UM-2 membrane filter (Amicon, Holland). A second gel filtration was conducted on a column (5 × 100 cm) of Sephadex G-75 (Calbiochem, USA) equilibrated with phosphate buffer, pH 6.5.

The homogeneity of the LGP isoenzymes was checked with the aid of electrophoresis in 7.5% PAAG in the presence of Na-DS.

The molecular masses of the enzymes were determined by disk electrophoresis in 15% PAAG in the presence of Na-DS by Laemmli's method [23] and by gel filtration on a column of TSK HW-65 gel (Toya Soda, Japan). Proteins were eluted with 0.01 Tris-HCl buffer, pH 6.5, fraction volume 0.35 ml. As markers we used cytochrome (12,400), soybean trypsin inhibitor (20,000), trypsin (24,000), carboanhydrase (30,000), ovalbumin (45,000), bovine serum albumin (67,000) and gelatin (95,000).

The isoelectric points of LGP-I and LGP-II were determined by analytical isoelectric focusing (IEF) in 7.5% PAAG with Pharmalites (Serva, FRG) or Ampholines (LKB, Sweden) in the pH range of 3.5-10 and 2.5-5 for 4 h. After the completion of IEF, the gels were cut into 2-mm disks, and each disk was placed separately in a test tube and was eluted in 1 ml of distilled water for 12 h, and then the LGP activity and the pH of each eluate were determined. In EP and IEF the gels were stained with a 0.75% solution of Coomassie G-250 (Calbiochem, USA) in 3.5% $HClO_4$ solution for 1 h, followed by the washing of the gel in 5% acetic acid. Oxidases were detected by staining the gels in a solution of 4-chloro-β-naphthol and from the decoloration of the polymeric dye Poly B-411 (LKB, Sweden).

The pH dependences of the enzymes were determined in 0.1 M Na tartrate buffer, pH 2.0-4.0 and Tris-acetate buffer, pH 4.0-8.0, and the temperature optima in 0.05 M sodium acetate buffer, pH 5.0. The heat stabilities of the lignin peroxidases were determined by incubating the enzymes at 20, 40, 60, and 80°C in a water thermostat.

The kinetic parameters of oxidation by the lignin peroxidases were determined in 20-200 μM veratryl alcohol in the presence of H_2O_2 in 0.1 M Na acetate buffer at pH 3.65.

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