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STUDY OF THE SUBSTRATE SPECIFICITIES OF THE EXTRACELLULAR LIGNIN PEROXIDASES OF THE WOOD-DESTROYING FUNGUS *Pleurotus ostreatus*

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The substrate specificities of two forms of purified extracellular lignin peroxidase isolated from a total enzyme preparation of the fungus Pleurotus ostreatus *— LGP-I and LGP-II — have been determined. The substrate specificities of the isoenzymes differ considerably: LGP-I preferentially destroys model compounds of lignin — the β-guaiacyl ether of 1-veratrylpropanol, coniferyl alcohol, and pyrocatechol, while LGP-II is most specific in relation to veratryl alcohol, veratrylpropane-1,3-diol, and vanillyl alcohol. Both forms partially oxidize syringaldazine and ABTS. The isoenzymes possess peroxidase and oxidase properties simultaneously, since veratryl, vanillyl, and coniferyl alcohols were oxidized by both forms of the enzyme only in the presence of H_2O_2, which confirms their peroxidase natures. At the same time, ABTS, syringaldazine, pyrocatechol, and o-phenylenediamine were also oxidized by the lignin peroxidase in the absence of H_2O_2, which confirms their oxidase function. The isoenzymes also possess Mn-peroxidase activity in relation to NADH. Since almost all substrates were oxidized by the enzymes only in the presence of hydrogen peroxide, they cannot be assigned to the class of oxidases. On the other hand, the LGP of* P. ostreatus *is not Mn-dependent, since the presence of manganese ions had no effect whatever on the oxidation of aromatic substrates by the enzyme. Moreover, both forms of the enzyme oxidized veratryl alcohol — a specific substrate for ligninases, which permits the extracellular isoenzymes of* P. ostreatus, *LGP-I and LGP-II, to be assigned to the class of ligninases.*

In 1983-1984, two research groups simultaneously reported the isolation of an enzyme, which was called ligninase (later, lignin peroxidase — LGP), from the fungus *Phanaerochaete chrysosporium* [1, 2]. It oxidized model dimers of lignin and polymeric lignin, both synthetic and native. The enzyme also catalyzed the oxidation of veratryl alcohol to veratraldehyde in the presence of hydrogen peroxide.

The authors suggested a "ping-pong" mechanism for this reaction: H_2O_2 reacts with the enzyme to form an intermediate which then reacts with the alcohol, liberating the enzyme.

Today, other reactions with the participation of LGP have also been discovered: the opening of an aromatic ring (**1**) [3, 4]; the $-C_\alpha-C_\beta-$ cleavage of arylglycerol aryl ethers (**2**) [5]; $-C_\beta-$O-4 cleavage (**3**) [6]; and the demethoxylation of lignin [7] and its oxidation to phenols [7].

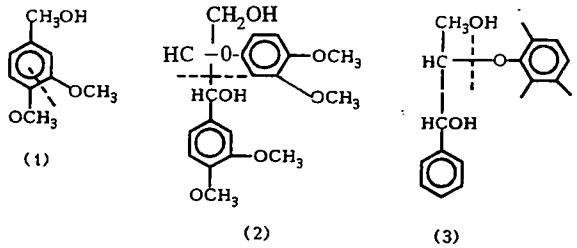

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TABLE 1. Substrate Specificity of the *P. ostreatus* LGP

Substrate	Specific activity, units/mg	
	LGP-I	LGP-II
Veratryl alcohol	13.9	17.4
β-Guaiacyl ether of 1-veratrylpropanol	4.7	3.7
1-Veratrulpropanol	1.7	5.4
Pyrocatechol	23.4	9.7
o-phenylenediamine	5.1	4.7
Coniferyl alcohol	3.5	2.2
Vanillyl alcohol	6.1	8.9
Syringaldazine	0.93	1.7
ABTS (2,2-azinobis(ammonium 3-ethylbenzothiazole-6-sulfonate)	1.4	2.8
NADH	24.6	26.3
DTBA (3,5-di-tert-butyl-4-hydroxyanisole)	10.9	12.3

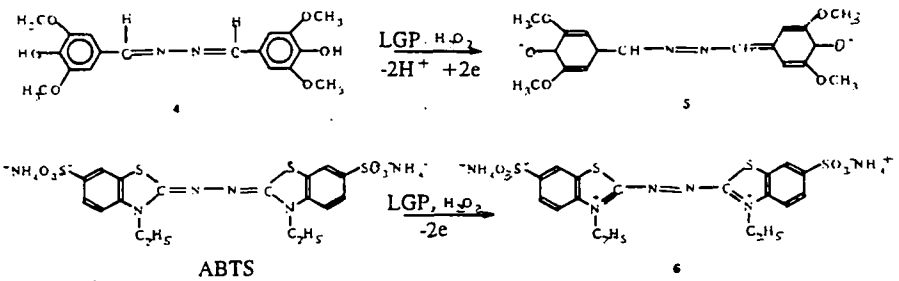

Fig. 1. Oxidation of syringaldazine and ABTS by the *P. ostreatus* lignin peroxidase.

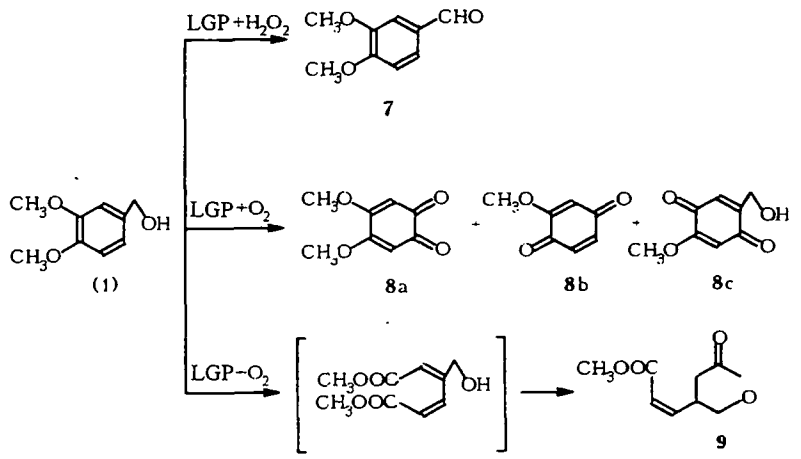

Fig. 2. Oxidation of veratryl alcohol by the LGP under aerobic conditions.

At first, the authors assumed that the *P. chrysosporium* ligninase was an individual protein [1, 3]; however, it was found subsequently that it consisted of a mixture of isoenzymes differing not only in their physicochemical and biochemical properties but also in their substrate specificities [4-8]. The number of isoenzymes ranges from 2 to 15, depending on the methods of isolation and purification, and also on the nature of the producing agent, the composition of the nutrient medium, and the time of cultivation.

It is known that, in relation to low-molecular-mass substrates, ligninolytic enzymes and their isoforms possess fairly broad specificities [9]. The specific substrate for lignin peroxidase is veratryl alcohol (1). However, a report has appeared that other enzymes of the ligninolytic complex, such as Mn-dependent peroxidase, as well, are capable of oxidizing this substrate [10]. The question of whether such an enzyme is a "ligninase" remains under discussion. The fact that an enzyme

oxidizes veratryl alcohol and may therefore be called a ligninase does not mean that it is capable of cleaving lignin. A different criterion is apparently necessary. A report has recently appeared that an oxidase function is an inherent characteristic of a ligninase. To answer the question concerned it is necessary to study the substrate specificities both of the most homogeneous enzymes and of their isoforms.

We have previously reported the capacity of a local strain of the basidial fungus *Pleurotus ostreatus* for forming ligninases on media containing various lignocellulose substrates [11, 12], the conditions for optimizing enzyme formation, and the isolation and purification of two forms of an extracellular lignin peroxidase — LGP-I and LGP-II — from the culture liquid of the fungus *P. ostreatus* [13, 14].

Continuing these investigations, we have studied the substrate specificities of the isoenzymes LGP-I and LGP-II formed by the fungus *P. ostreatus* UzBI-I108.

The substrate specificities of the lignin peroxidases were investigated on eleven low-molecular-mass substrates. Their selection was determined by the following considerations: in the first place, those substrates were selected that are usually employed for measuring the activities of ligninolytic enzymes: veratryl alcohol — a substrate for ligninases — pyrocatechol, syringaldazine, ABTS (2,2-azinobis(ammonium 3-ethylbenzothiazoline-6-sulphonate)), and *o*-phenylenediamine — substrates for laccases and peroxidases — and NADH — a substrate for Mn peroxidases: in the second place, we used various model compounds of lignin: the β-guaiacyl ether of 1-veratrylpropanol, 1-veratrylpropanediol, and coniferyl and vanillyl alcohols. The demethoxylating activity of the lignin peroxidases was also determined with the aid of 3,5-di-*tert*-butyl-4-hydroxyanisole (DTBA).

The experiments showed that both forms of the enzyme possessed the capacity for partially oxidizing syringaldazine (**4**) with the formation of tetramethoxyazobis(methylenecyclohexadienonyl) (**5**) (Fig. 1). On the oxidation of ABTS a fairly stable cation radical was formed — the azo compound (**6**) [16]. Coniferyl and veratryl alcohols were oxidized by the LGP to the corresponding aldehydes, and vanillyl alcohol to vanillin. Phenolic compounds (pyrocatechol) were oxidized by the LPG in the presence of O_2 to quinones, while the oxidation of *o*-phenylenediamine led to the formation of an *o*-quinone imine [15].

The results presented in Table 1 show that LGP-I preferentially destroys model compounds of lignin — the β-guaiacyl ether of 1-veratrylpropanol, coniferyl alcohol, and pyrocatechol, while LGP-II is most specific for veratryl alcohol, veratrylpropane-1,3-diol, and vanillyl alcohol. Figure 2 shows the specific reactions involved in the oxidation of veratryl alcohol by the LGP of the fungus *P. ostreatus*. On the oxidation of veratryl alcohol by the LPG in the presence of H_2O_2 dehydrogenation of the alcohol molecule takes place with the formation of veratraldehyde (**7**), while in the presence of O_2 the LGP catalyzes various enzymatic reactions [16]. Thus, for example, dehydroxylation and demethoxylation of the substrate take place with the formation of quinones (**8**a,b,c) and the cleavage of the aromatic ring with the formation of lactones (**9**). The oxidation of vanillyl alcohol with the LGP in the presence of H_2O_2 forms at least four products of oxidative catalysis, such as vanillin, vanillic acid, 1,2-dihydroxy-3-methoxybenzene and 3-methoxy-*p*-quinone.

The investigations of the substrate specificities of the isoenzymes of the *P. ostreatus* ligninase showed that the enzyme oxidized all the substrates that we used. Table 1 gives the specific activities in relation to the various substrates obtained for LGP-I and LGP-II. A number of the substrates — veratryl, vanillyl, and coniferyl alcohols, *o*-phenylenediamine, pyrocatechol, DTBA, and the model dimers of lignin — were oxidized by the LGP only in the presence of H_2O_2. This property of the enzyme confirmed its peroxidase nature. However, ABTS and syringaldazine were oxidized by the *P. ostreatus* enzymes even in the absence of H_2O_2 but in the presence of O_2, which showed the existence of an oxidase function in the ligninase. The addition of hydrogen peroxide to the reaction mixture increased the rate of oxidation of these substrates. It follows from the literature that ligninases may possess an oxidase capacity [17].

In relation to DTBA, LGP-I and LGP-II exhibited a demethoxylating activity, oxidizing the substrate to 3,5-di-*tert*-butyl-*p*-benzoquinone. On the oxidation of model compounds of lignin — the β-guaiacyl ether of 1-veratrylpropanol and 1-veratrylpropanediol — cleavage of the $C_\alpha - C_\beta$ bond took place, since one of the reaction products was veratraldehyde, the formation of which was recorded with the aid of reversed-phased chromatography.

The *P. ostreatus* LGPs also exhibited Mn peroxidase activity, oxidizing NADH (see Table 1). However, they did not possess another characteristic property of Mn peroxidases: the addition of Mn^{2+} to the reaction mixture did not affect the activity of the isoenzymes in relation to other substrates. The property of oxidizing substrates characteristic for Mn peroxidase (NADH) has also been reported for the *Ph. chrysosporium* ligninase [18].

Thus, it has been shown in this paper that the LGP isoenzymes differ considerably in substrate specificity. Both enzymes partially oxidize syringaldazine and ABTS and possess peroxidase and oxidase capacities simultaneously.

On the whole, the *P. ostreatus* LGP possesses both oxidase properties and Mn peroxidase activity in relation to NADH, which makes its identification difficult. Since both forms of the enzyme oxidized veratryl alcohol, a specific substrate for ligninases, the extracellular isoenzymes of *P. ostreatus*, LGP-I and LGP-II, may be assigned to the class of ligninases.

EXPERIMENTAL

The local strain of *Pleurotus ostreatus*, UzBI-I108, was isolated from a poplar trunk. The fungus was sown from slope wort-agar media into a sterilized nutrient medium containing 0.3% of wort and 1% of an aqueous extract of depleted cottonseed oilcake pulp obtained by subjecting the substrate (ground in a ball mill to particle dimensions of 0.01-0.1 mm) to high pressures in a IBFM press (a hydraulic press, developed by workers in the Institute of the Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences). Cultivation was conducted by the deep method at 28-29°C in 500-ml Erlenmeyer conical flasks containing 200 ml of nutrient medium for 3-30 days on circular shaking machines at 250 rpm.

The activity of the LGP was determined spectrophotometrically from the rate of oxidation of 3,4-dimethoxybenzyl (veratryl) alcohol to veratraldehyde [19]. The method is based on the increase in absorption at 310 nm (absorption maximum of veratraldehyde, E_{310} = 9300 M^{-1} cm^{-1}). Composition of the reaction mixture: 0.1 M Na tartrate buffer, pH 3.0; 0.4 mM H_2O_2; 2 mM veratryl alcohol; the enzyme preparation in an amount ensuring an increase in optical density by 0.4 unit/min. The total volume of the reaction mixture was 3 ml. The absorption kinetics were measured on a Shimadzu (Japan) scanning double-beam spectrophotometer for 3 min. As the unit of activity we took the amount of enzyme necessary for the formation of 1 μmole of veratraldehyde per minute per 1 ml (mg) of enzyme at 20°C.

The substrate specificities of the enzymes were determined with the use of purified preparations of LGP-I and LGP-II. The reaction mixture included: 0.1 M Na acetate buffer, pH 4.0; 0.05 M H_2O_2; 0.1 mM substrate and the enzyme. The activities of the enzymes in relation to all the substrates were determined spectrophotometrically at various wavelengths: for the model dimers of lignin: 310 nm (E_{310} = 9300 M^{-1} cm^{-1}; pyrocatechol: 410 nm (E_{410} = 740 M^{-1} cm^{-1}); coniferyl alcohol) 338 nm (E_{338} = 14,200 M^{-1} cm^{-1}); vanillyl alcohol: 300 nm (E_{300} = 5750 M^{-1} cm^{-1}); syringaldehyde: 525 nm (E_{525} = 65,000 M^{-1} cm^{-1}); ABTS: 415 nm (E_{415} = 31,100 M^{-1} cm^{-1}); and DTBA: 258 nm (E_{258} = 11,500 M^{-1} cm^{-1}).

The products of the enzyme-catalyzed transformation of the substrates were analyzed by TLC on Silufol UV-254 plates 10 cm high. The products were extracted with ethyl acetate 3-5 times, the extracts were combined, and the organic phase was washed with distilled water and evaporated to dryness in a rotary evaporator. The dry residue was dissolved in ethyl acetate and subjected to TLC. The enzyme treatment of the model compounds of lignin was conducted in hermetically sealed glass bottles in an atmosphere of oxygen at 36°C for 18-24 h. The products of the reactions with model compounds of lignin were separated in the solvent system hexane–chloroform–ethyl acetate–acetic acid (1:1:1:0.1) and were identified by chromato-mass spectrometry.

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