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Isolation and Purification of Enzymes from Ligninolytic Complex of the Basidial Fungus *Trametes pubescens* (Schumach.) Pilát and Study of Their Properties

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Abstract—A method for purification of enzymes from the ligninolityc complex of the basidiomycete *Trametes pubescens* (Schumach.) Pilát has been elaborated. Two homogeneous isoforms of laccases (laccase 1 and laccase 2) as well as a homogeneous preparation of lignin peroxidase were isolated. Basic biochemical parameters of the enzymes were determined, such as the molecular weights (67, 67, and 45 kD, respectively), isoelectric points (5.3, 5.1, and 4.2, respectively), as well as content and composition of the carbohydrate moiety of the laccases (N-acetylglucosamine, mannose, and xylose). The pH dependences and thermal stabilities of the laccases were investigated. The kinetic parameters of the enzymatic reactions catalyzed by the laccases were determined using different substrates, such as catechol, hydroquinone, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate), and $K_4Fe(CN)_6$. The structure of the active sites of both laccases and the lignin peroxidase were studied by EPR, CD, and UV-VIS spectroscopy, as well as using fluorescence analysis. Our studies showed similarity of the spectral characteristics of the two laccases, whereas their kinetic properties were found to be different.

Key words: *Trametes pubescens*, laccase, lignin peroxidase, spectral properties

The ligninolytic complexes of wood destroying fungi usually include various types of enzymes [1].

The first type of the enzymes includes heme-containing oxidoreductases: lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) reducing hydrogen peroxide via the two-electron pathway to yield water. The characteristic feature of LiP is its ability to oxidize dimethoxyphenyl substrates yielding intermediate cation radicals. The other representative of these enzymes, MnP, can catalyze oxidation of the non-phenolic lignin structures only in the presence of Mn^{2+}. The product of this enzymatic reaction, Mn^{3+}, in the complex with anions of dicarboxylic acids is a high potential mediator. Many investigators consider MnP as the second most important enzyme of the ligninolytic complex after LiP. The main difference in the reactions catalyzed by LiP and MnP is the terminal reaction of the peroxidase catalytic cycle leading to the reduction of compound II to the native enzyme [2].

The second type includes laccases (Lc) playing an important role in the development and morphogenesis of fungi and plants. Also, laccases together with LiP and MnP play an important role in lignin metabolism, gumification of organic substances, and detoxification of xenobiotics.

Laccase (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2) catalyzes oxidation of ortho- and para-diphenols, aminophenols, polyphenols, polyamines, phenolic lignin structures, as well as some inorganic ions with the reduction of molecular oxygen to water [3-5]. The enzyme contains four copper ions of three types: T1, T2, and T3 [3]. The type 1 copper (T1) imparts to the enzyme blue color due to an absorption band in the visible region of the spectrum (610 nm) and exhibits small values of the

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hyperfine structure splitting of the low field EPR spectrum ((80-95)·10^{-4} cm^{-1}). The mononuclear copper-containing site (T2) is not detectable spectrophotometrically, but the EPR spectrum of the enzyme exhibits a signal typical for the low molecular weight copper complexes (line splitting values, (180-200)·10^{-4} cm^{-1}). The binuclear copper-containing site (T3) in which two bivalent copper ions coupled antiferromagnetically through the hydroxide bridge imparts absorption at 330 nm, but it cannot be detected by EPR spectroscopy.

The 3rd type of the enzymes of the ligninolytic complex of wood-destroying fungi includes flavin-containing enzymes. They perform mainly accessorial functions: two-electron reduction of an oxygen molecule to hydrogen peroxide and two-electron oxidation of the hydroxyl group of a carbohydrate molecule of aliphatic or aromatic alcohol. The representative of this group is cellobiose dehydrogenase containing flavin and heme as the prosthetic groups.

For the last decade, investigations have been directed to searching for and study of new strains of Basidiomycetes (producers of laccases) and finding of enzymes with new physical and chemical properties, this being due to the possibility of wide application of these enzymes (production of bioplastics, biofuel, biodegradation of xenobiotics, organic synthesis, etc.) [6-9].

The basidiomycete *Trametes* (*Coriolus*) *pubescens* produces several extracellular laccases and peroxidases [10, 11]. It is also known as a medicinal fungus. The mycelium and fruit bodies of *T. pubescens* are used for extraction of polysaccharides bound to proteins possessing antitumor and immunomodulating activities [12]. The strain *T. pubescens 923-2* is a producer of biomass, a substance for isolation of bioactive nutrient [13]. After the removal of the biomass, the filtrate of the culture liquid can serve as the source for isolation of extracellular enzymes.

The goal of the present work was to investigate the physical and chemical properties of the enzymes of the ligninolytic complex of the fungus *T. pubescens* and the possibility of the subsequent use of these enzymes in biotechnology.

MATERIALS AND METHODS

Strain and growth conditions. The strain *Trametes pubescens* (Schumach.) Pilát (Syn.: *Coriolus pubescens* (Schum. ex Fr.) Quél.) VSB 923-2 was obtained from the collection of the State Research Institute of Biosynthesis of Protein Substances (Moscow) and has been maintained in the collection of the author (E. S. Gorshina) for 20 years in tubes with wort agar slants at 4°C with reseeding once a year.

The producing strain was cultivated in 30-liter fermenter (Marubishi, Japan) with working volume of 20 liters at excess pressure of 0.4 atm. Aeration with oxygen (1 liter/liter per min) was performed under sterile conditions with mechanical stirring (250 rpm) with a turbine stirrer. The medium was prepared using tap water and contained (in g/liter): glucose, 20.0; $(NH_4)_2SO_4$, 2.5; KH_2PO_4, 1.2; urea, 0.7; concentrated corn extract, 10. The fungus was cultivated at constant pH 3.5 and 26°C. During the cultivation, the following parameters were determined: temperature, pH, content of dissolved oxygen (% saturation), content of substrate (glucose) (as the content of reducing substances), concentration of biomass, and oxidase activity (spectrophotometrically using catechol and hydroquinone). The seeding material (10% of the volume) was grown in 750-ml flasks containing 150 ml of the medium in a shaker at 180 rpm and 26°C. The time of cultivation was 114 h. The maximum of the oxidase activity was observed at 102 h, in the stationary phase that started after 60 h of growth. The biomass was separated by filtration through polyamide material.

Isolation and purification of enzymes. Low-pressure column chromatography was performed using standard equipment for low-pressure liquid chromatography (Pharmacia LKB Biotechnology, Sweden) at 4°C. Medium- and high-pressure liquid chromatography were performed using a Staier HPLC system (Akvilon, Russia) at 20°C.

Determination of molecular weight and isoelectrofocusing. Molecular weight was determined by gradient SDS-PAGE as described in [14] using cellulase, BSA, ovalbumin, carboanhydrase, trypsin inhibitor, and lysozyme as the protein standards (94.6, 66.2, 45.0, 31.0, 21.5, and 14.4 kD, respectively). Isoelectrofocusing was performed as described in [15] using ampholines of pH 3.8-9.3 (Pharmacia LKB Biotechnology) and Model 111 Mini IEF cells (BioRad, USA).

Spectral assays. Spectrophotometric measurements were performed using a Hitachi-557 spectrophotometer (Hitachi, Japan).

The activity of Lc during the isolation and purification, as well as during the investigation of thermal stability of the enzymes, was measured spectrophotometrically [16, 17], using 10 mM catechol as the chromogenic substrate (λ = 410 nm, ε = 740 $M^{-1}\cdot cm^{-1}$) in 0.1 M Na-citrate-phosphate buffer, pH 4.5. The kinetic parameters for the reaction of oxidation of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate (ABTS) catalyzed by Lc1 and Lc2 were determined at 436 nm using the molar absorption coefficient of the oxidation product ε = 36,000 $M^{-1}\cdot cm^{-1}$. Before the experiments, the preparations of Lc were incubated at room temperature for 3 h.

The activity of LiP during the isolation and purification was determined spectrophotometrically [18] in the presence of 8 mM ABTS (λ = 436 nm, ε = 36,000 $M^{-1}\cdot cm^{-1}$) and 1 mM H_2O_2 in 0.1 M Na-acetate buffer, pH 4.5, under anaerobic conditions.

The activity of MnP was determined as described in [19] using Mn^{2+} as the substrate. The reaction mixture

contained the enzyme, 0.1 M Na-tartrate buffer (pH 5.0), 0.1 mM H_2O_2, and 0.1 mM $MnSO_4$. The product (Mn(III)) forms a meta-stable complex with tartrate exhibiting characteristic absorbance at 238 nm (ε = 6500 $M^{-1}\cdot cm^{-1}$).

The activity of cellobiose dehydrogenase was measured by the reduction of dichlorophenolindophenol in the presence of cellobiose (λ = 520 nm, ε = 6300 $M^{-1}\cdot cm^{-1}$) [20]. All kinetic parameters and enzyme activities were measured at 20°C. Protein concentration was determined by the method described in [21] by the difference in the optical density of the protein solution at 228.5 and 234.5 nm using a corresponding calibration curve.

Absorption spectra were recorded using a DU 650 Coulter spectrophotometer (Beckman, Germany) in 1-cm cuvettes at 120 nm/min. Fluorescence emission and excitation spectra were recorded on an RF-5301 PC spectrofluorimeter (Shimadzu, Japan) at 120 nm/min and slit width of 5 nm. EPR spectra were recorded using an ESC-106 EPR-spectrometer (Bruker, Germany). CD spectra were taken on a J-715 spectrophotometer (Jasco, Japan) in a 0.1-cm cuvette at 20 nm/min and slit width of 1 nm. Parameters of the secondary structure were calculated using the Belok computer program (Russia).

Kinetic measurements and pH dependence. The catalytic parameters of the enzymatic reactions catalyzed by Lc for catechol, hydroquinone, and $K_4Fe(CN)_6$, as well as the dependence of the enzyme activity on pH were measured using a Clark-type oxygen electrode by the changes in concentration of molecular oxygen in the system during the enzymatic oxidation of the donor substrate. The constants were calculated considering the stoichiometry of the reaction. The original concentration of dioxygen was taken as 0.26 mM. Commercial preparations of catechol and hydroquinone were purified by their sublimation under vacuum.

Determination of metal content. Metal content in Lc preparations was determined as described in [22], using 0.1% biquinoline solution in glacial acetic acid as the chelator, and also by laser mass-spectrometry on a Lasma laser mass-spectrometer (Institute of Space Research, Russian Academy of Sciences) [23].

Assay of the carbohydrate moiety of enzymes. The composition of the carbohydrate part of enzymes was determined by ion-exchange chromatography with the detection of Cu-bicinchoniate using an LC 2000 unit (Biotronic, Germany). The samples were incubated in 4 M CF_3COOH for 3 h at 110°C for protein hydrolysis.

Chemicals. Toyopearl DEAE-650M (Tosoh, Japan); 2-mercaptoethanol (Ferak, Germany); ampholines (Pharmacia LKB Biotechnology, Sweden); Coomassie blue R-250, protein standard kits for gel chromatography and electrophoresis (Serva, Germany); Tris, glycine, acrylamide, N,N′-methylenebisacrylamide, ammonium persulfate, $K_4Fe(CN)_6$ (ICN, USA); Servacel DEAE-52 (Reanal, Hungary); 2,2′-biquinoline, Cu-bicinchoniate, catechol, hydroquinone, guaiacol, ABTS (Sigma, USA); Na_2HPO_4 (Merck, Germany); glycerol, methanol, ethanol, CH_3COOH, CF_3COOH, HCl, H_3PO_4, KH_2PO_4, $(NH_4)_2SO_4$, NaOH, $MgSO_4$, $MnSO_4$, $ZnSO_4$, $CuSO_4$, peptone, and glucose were domestic preparations of analytical grade. All solutions except for those for fermentation were prepared using water purified on a MilliQ unit (Millipore, USA).

RESULTS AND DISCUSSION

A method for isolation and purification of enzymes of the ligninolytic complex from the basidial fungus *T. pubescens* has been developed. The method includes the following steps: precipitation of the proteins from the culture liquid with ammonium sulfate (0-90% saturation), low pressure ion-exchange chromatography on Servacel DEAE-52 and Toyopearl DEAE-650M, Bio-Gel HTP adsorption chromatography, HPLC on a Bio Sep-SEC-S 2000 column (Phenomenex, USA), and ion-exchange chromatography on a TSK DEAE-2SW column (Pharmacia LKB Biotechnology). The procedure resulted in isolation of a homogeneous preparation of extracellular blue laccase (Lc1) with the specific activity of 66.7 μmol/min per mg protein and also a complex of enzymes possessing oxidase and peroxidase activities. The preparations of the second isoform of laccase (Lc2, specific activity 75.1 μmol/min per mg protein) and peroxidase (LiP, 15.5 μmol/min per mg protein) were obtained using medium-pressure hydrophobic chromatography on a Phenyl-Superose FPLC column (Pharmacia LKB Biotechnology).

Two of the three purified enzymes possessed oxidase activity in the reaction of oxidation of ABTS, hydroquinone, and catechol, and the third enzyme exhibited peroxidase activity in the reaction of oxidation of ABTS and guaiacol with hydrogen peroxide under anaerobic conditions in the absence of Mn^{2+}.

During the purification of the enzymes from the culture liquid, no cellobiose dehydrogenase activity was detected and only traces of Mn-dependent peroxidase activity were observed. Thus, the strain of *T. pubescens* used in the present work under the described conditions of cultivation produced two ligninolytic enzymes, Lc and LiP.

It was shown that Lc1 and Lc2 from *T. pubescens* are monomeric enzymes of similar molecular weights (67 kD). Both isolated Lc are glycoproteins, and their carbohydrate part constitutes about 13% of the protein weight. The carbohydrate part of Lc from *T. pubescens* is composed of N-acetylglucosamine, mannose, and xylose. LiP is a protein of 45 kD. The enzymes Lc1, Lc2, and LiP are acidic proteins with p*I* values of 5.3, 5.1, and 4.2, respectively. The biquinoline method and laser mass-spectrometry assay showed that the average content of copper in Lc1 and Lc2 from *T. pubescens* was 3.9 and 3.8

metal ions per protein molecule, respectively, this being typical for blue oxidases [3, 5, 7, 10, 11].

The presence of LiP in the culture liquid of the basidiomycete was previously determined by the immunochemical approach [10]. The isolated peroxidase did not exhibit Mn-peroxidase activity, and in contrast to MnP catalyzed H_2O_2-dependent oxidation of ABTS, guaiacol, and catechol in the absence of Mn^{2+} under anaerobic conditions, this being characteristic for LiP and horseradish peroxidase. Based on these results and literature data, we conclude that the isolated protein belongs to the family of lignin peroxidases.

To study the structure of the active site of the oxidoreductases from *T. pubescens*, the spectral properties of the enzymes were investigated. The absorption spectra of Lc1 and Lc2 were typical for native blue Lc of Basidiomycetes. They exhibited signals of both copper-containing sites (T1 (peak at 610 nm) and T3 (shoulder at 330 nm)) (Fig. 1a). Among the known fungal laccases, only Lc from the fungus *Phlebia radiata* has no T3 copper and contains PQQ (pyrroquinoline quinone) as the cofactor [24]. The absorption spectrum of LiP is typical for heme-containing enzymes: it exhibits a pronounced absorption maximum at 407 nm (Soret band) and absorption bands at 500 and 633 nm (Fig. 1b).

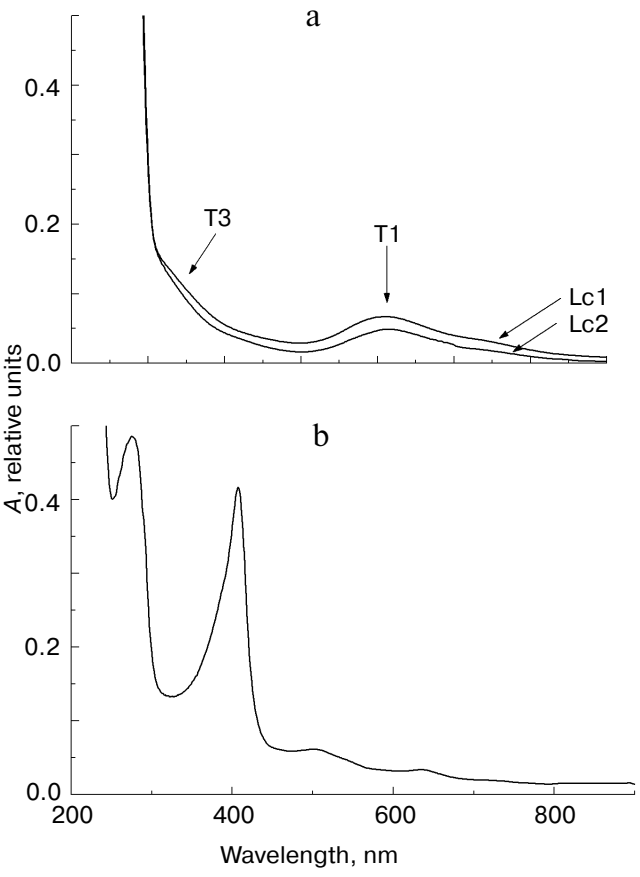

Fig. 1. Absorption spectra of Lc1 and Lc2 (a) and LiP (b) from the basidiomycete *T. pubescens*. The sample contained 1 mg/ml of laccase or 0.42 mg/ml ligninoperoxidase in 0.05 M phosphate buffer, pH 6.5, path length 1 cm. The spectra were taken at 120 nm/min at 20°C.

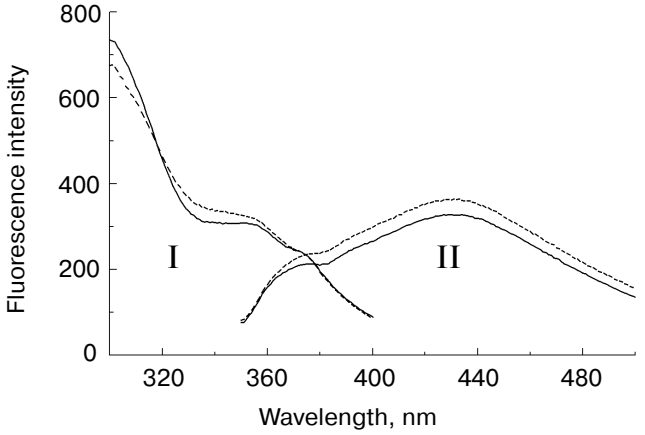

Fig. 2. Excitation (I) and emission (II) fluorescence spectra of Lc1 (dashed lines) and Lc2 (solid lines) from the basidiomycete *T. pubescens*. The sample contained 1 mg/ml of laccase or 0.42 mg/ml lignin peroxidase in 0.05 M phosphate buffer, pH 6.5. The spectra were taken at 120 nm/min at 20°C.

Excitation in the region of the binuclear copper-containing site (330 nm) results in the emission at 429 and 431 nm for Lc1 and Lc2, respectively (Fig. 2). As seen from Figs. 1a and 2, the absorption spectra and the fluorescence excitation and emission spectra of Lc1 and Lc2 are virtually the same.

The EPR spectra of Lc1 and Lc2 are virtually identical, being typical for the blue laccases (Fig. 3a, *1* and *2*). The values for the component g-tensor and A-tensor (hyperfine interaction) are the following: for T1, $g_{||} = 2.20$ and $A_{||} = 90 \cdot 10^{-4}$ cm^{-1}; for T2, $g_{||} = 2.25$ and $A_{||} = 188 \cdot 10^{-4}$ cm^{-1}. Thus, although there are some differences in the spectral parameters of the investigated enzymes, the general character of the active site structure of the blue laccases containing four copper ions is similar.

The EPR spectrum of LiP (Fig. 3b) exhibited a signal at $g \cong 6.0$, which is characteristic for high-spin iron (Fe^{3+}) in heme-containing enzymes [25].

Secondary structure of the enzymes was studied using CD spectroscopy. It was shown that Lc1 and Lc2 have insignificant differences in their secondary structure (Fig. 4). They contain ~10% α-helical structures and 30% β-structures, ~40% of the protein being random coil structures. The CD spectrum of LiP significantly differs from the Lc spectra (Fig. 4). It has a clear absorption maximum at 210 nm and a shoulder at 222 nm, this being in agreement with the literature data [26, 27].

The table presents the catalytic constants (k_{cat}) of the enzymatic reactions for different donor substrates of Lc1 and Lc2, Michaelis constants (K_m), as well as the ratio of these values that characterizes the efficiency of the cat-

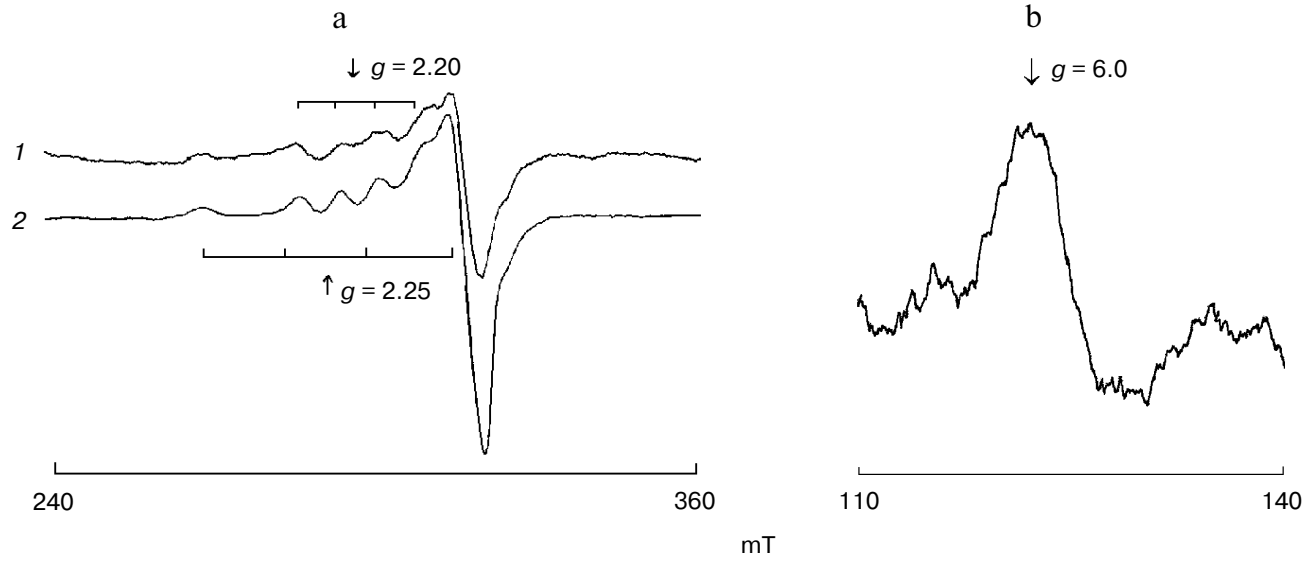

Fig. 3. EPR spectra of the enzymes of the ligninolytic complex from the basidiomycete *T. pubescens* (0.05 M phosphate buffer, pH 6.5, 77°K). a) EPR spectra of Lc1 (*1*) and Lc (*2*). Concentration of Lc was 1 mg/ml; center field, 308 mT; field scanning, 150 mT; modulation amplitude, 0.5 mT; SHF power, 20 mV; amplification, $1 \cdot 10^5$ (*1*) and $4 \cdot 10^5$ (*2*). b) EPR spectrum of LiP of *T. pubescens*. The concentration of LiP was 0.42 mg/ml; center field, 160 mT; field scanning, 80 mT; modulation amplitude, 1.14 mT; SHF power, 50 mV; amplification, $1 \cdot 10^5$.

alytic process. The substrates of laccases can be divided into two groups, electron donors and donors of hydrogen atoms. We used both groups of the substrates: ABTS and $K_4Fe(CN)_6$ belongs to the first group, and hydroquinone and catechol to the second group. It was shown that the laccases of *T. pubescens* catalyze oxidation of both groups of substrates.

The optimal pH value for the activity of both Lc while using catechol as the substrate was virtually the same (4.0-4.5). In the case of $K_4Fe(CN)_6$, the activity of the enzymes does not change with the increase in pH of the solution from 2.5 to 6.0, and then decreases gradually with subsequent increase in pH.

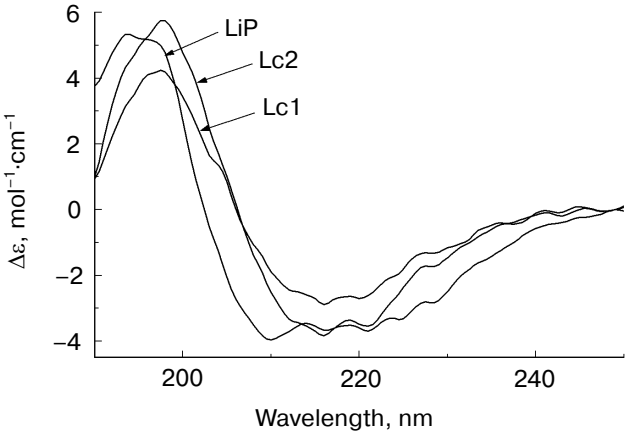

Fig. 4. CD spectra of Lc1, Lc2, and LiP from *T. pubescens*. The cuvette (0.1-cm path length) contained 1 mg/ml of laccase or 0.42 mg/ml of lignin peroxidase in 0.05 M phosphate buffer, pH 6.5. The spectra were taken at 20 nm/min at 20°C.

The differences in the pH dependences for the substrates of Lc belonging to different types of donors can be explained in the following way. For both types of substrates, the steady state rate of the enzymatic reactions decreases with increase in pH due to the inhibition of the reaction by OH^- that binds to the trinuclear T2/T3-cluster of laccases [1]. For catechol, which is a phenolic substrate of laccases, the ionization potential decreases with increasing pH, and consequently the reaction rate increases. Superposition of these two factors results in a bell-shaped pH dependence of the enzymatic reaction for the hydrogen donor substrates. In the case of $K_4Fe(CN)_6$, which is an electron donor, the ionization potential does not change with changes in pH. As a result, the pH dependence exhibits a plateau with subsequent gradual decrease in the reaction rate.

Incubation of Lc1 from *T. pubescens* at 37°C resulted in the loss of only ~25% of the original activity in 72 h, while Lc from the basidiomycete *Trametes hirsuta* described in the literature loses ~40% of its activity. Hence, the Lc1 from *T. pubescens* exhibits a rather high thermal stability, which can be useful for its application in the biotechnological processes.

Thus, from the culture liquid of the basidiomycete *T. pubescens*, two forms of laccases and lignin peroxidase were purified to homogeneous state and characterized. During the purification, no cellobiose dehydrogenase activity was detected, and only traces of the Mn-peroxidase activity were found. In contrast to Lc1, Lc2 forms a

Kinetic parameters of Lc1 and Lc2 from the basidiomycete *T. pubescens*

Substrate	Lc1			Lc2		
	K_m, mM	k_{cat}, sec^{-1}	$(k_{cat}/K_m) \cdot 10^4$, $sec^{-1} \cdot M^{-1}$	K_m, mM	k_{cat}, sec^{-1}	$(k_{cat}/K_m) \cdot 10^4$, $sec^{-1} \cdot M^{-1}$
Hydroquinone	0.50	240	48	0.2	170	85
Catechol	0.20	60	30	0.6	160	26
ABTS	0.05	150	300	0.06	400	670
$K_4Fe(CN)_6$	0.7	280	40	0.2	370	185

Note: Constants were calculated as the mean values of three measurements. The error of measurement did not exceed 10%. Conditions: 0.1 M Na-citrate-phosphate buffer, pH 4.5, 20°C.

rather firm complex with lignin peroxidase that was destroyed only with the use of hydrophobic chromatography in the final step of the purification. The laccases from *T. pubescens* compared to the laccases from other Basidiomycetes exhibit a higher thermal stability. This fact together with the ability of Lc2 to form a firm enzyme complex with LiP makes it possible to use these enzymes together with mediators in lignification of paper pulp.

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