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Isolation and Study of Some Properties of Laccase from the Basidiomycetes *Cerrena maxima*

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Abstract—A new strain producing extracellular laccase (*Cerrena maxima* 0275) was found by screening of isolates of Basidiomycetes, and the dynamics of laccase biosynthesis by this strain was studied. The enzyme was purified to homogeneity. The molecular weight of the enzyme is 57 kD, and its p*I* is 3.5. The activity is constant at pH values in the range 3.0-5.0. The temperature optimum for activity is 50°C. The thermal stability of the laccase was studied. The catalytic and Michaelis constants for catechol, hydroquinone, sinapinic acid, and $K_4Fe(CN)_6$ were determined. The standard redox potential of type 1 copper in the enzyme is 750 ± 5 mV. Thus, the investigated laccase is a high redox potential laccase.

Key words: *Cerrena maxima*, laccase, redox potential, pH optimum, isoelectric point, Michaelis constant

The activation of molecular oxygen is an important reaction in biological systems. Enzymes capable of activating oxygen comprise two families—oxidases and oxygenases [1]. Copper-containing oxidases are an important class of enzymes catalyzing the reduction of molecular oxygen by four electron transfers yielding water. The reaction proceeds without formation of hydrogen peroxide and is coupled to the oxidation of an electron donor substrate by the single electron mechanism [2, 3]. Blue copper-containing proteins perform intramolecular electron transfer with rates that are necessary and sufficient for catalytic reduction of oxygen [3, 6]. This increases interest in studies of laccase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase EC 1.14.18.1)—blue copper oxidase containing four copper ions in the active site [7, 8]. Prosthetic copper ions are subdivided into three basic types: the first type (blue copper) is designated T1, the second type T2, and the third type T3 [3, 8] representing a binuclear copper site. The function of T1 sites in the enzyme involves electron transfer from a substrate to a three-atom copper cluster. The redox potential E^o of the T1 site of laccase from *Rhus vernicifera* is 344 mV versus the standard hydrogen electrode (344 vs. SHE) [8]. However, the corresponding value for fungal laccases is significantly higher, 500-770 mV [1, 3, 5, 9]. It was found previously that the efficiency of the catalysis depends linearly on the redox potential of type 1 copper in the active site of the enzyme [10]. That is why laccases with high redox potential copper sites are of special interest. Comparative study of these enzymes should reveal similarities and differences in the mechanism of their functioning.

The goal of the present work was to isolate and purify the extracellular laccase from the basidium fungus *Cerrena maxima* and to characterize the enzyme including determination of the redox potential of its type 1 copper ion.

MATERIALS AND METHODS

The Basidiomycetes collection of the Komarov Botanical Institute of the Russian Academy of Sciences (LENBIN) was used to select strains producing extracellular laccase of high activity. Cultures were grown as described in [11].

Laccase isolation and purification. After nine days of submerged cultivation of *C. maxima* 0275, the culture liquid containing extracellular laccase was filtered to remove the mycelium. The filtrate was concentrated by ultrafiltration using an AR02 15PA device (Russia) equipped with a membrane retaining polymers (proteins) with molecular weight exceeding 15 kD. The enzyme was further purified by iso-

Abbreviations: E^o) standard redox potential; λ) wavelength; ε) molar absorption coefficient.

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electric focusing of the culture liquid concentrate in a sucrose gradient containing ampholines providing a pH gradient from 2.5 to 5.0. The ampholines and the column used were from Pharmacia (Sweden). The active enzyme fractions were collected and dialyzed against 5 mM potassium phosphate buffer, pH 7.2. Then the enzyme solution was concentrated in an Amicon concentrating cell (USA) using PM 10 or PM 25 membranes and was frozen to −18°C. Homogeneous preparation of the enzyme was obtained by FPLC on a Superdex 200 column (Pharmacia, Biotech, Sweden) equilibrated with 15 mM potassium phosphate, pH 6.5. The enzyme was eluted with the same buffer at 1 ml/min.

The molecular weight of the laccase was estimated by electrophoresis in 12.6% polyacrylamide gel in the presence of 10% SDS [12].

Laccase activity was measured spectrophotometrically using a Hitachi 557 spectrophotometer (Japan) [11]. The catalytic parameters of the reaction (K_m and k_{cat}) were calculated from the initial velocity values of the enzyme reaction. Hydroquinone (Merck, Germany) (λ = 247 nm, ε = 20,000 $M^{-1}\cdot cm^{-1}$), sinapinic acid (Merck) (λ = 307 nm, ε = 16,000 $M^{-1}\cdot cm^{-1}$), catechol (Fluka, Germany) (λ = 410 nm, ε = 740 $M^{-1}\cdot cm^{-1}$), and $K_4Fe(CN)_6$ (Fluka) (λ = 420 nm, ε = 1045 $M^{-1}\cdot cm^{-1}$) were used as substrates.

The pH optimum for the laccase reaction was determined in the range pH 3.0-7.0 using catechol as the substrate and a universal buffer system with step of 0.5.

Stability of the enzyme was studied at 40°C in 0.1 M sodium acetate buffer, pH 4.5. Enzymatic activity was measured using catechol as described above every 2 h for the first 24 h, then every 12 h for the next 2 days.

Temperature optimum was measured using catechol as described above in 0.1 M sodium acetate buffer, pH 4.5.

The redox potential of the T1 site of the laccase was determined by titration as is usually employed for measuring redox potentials of laccase copper sites [8, 10]. Potassium octocyanomolybdate was used as a mediator [13, 14].

A 0.02-μmole portion of laccase (0.2 ml of concentrated enzyme solution) was added into the cell containing 2 μM $K_3Mo(CN)_8$, 0.4-20 μM $K_4Mo(CN)_8$, and also the ferri/ferrocyanide couple in the ratio 100 : 1 in 0.1 M phosphate buffer, pH 6.0 (the final volume of the solution was 2 ml.) To provide anaerobic conditions, the cell was purged with pure argon before and during the addition of the enzyme. The gas was purified from oxygen impurities by passing it through a system of solutions containing VCl_2 [15]. After the achievement of the redox equilibrium, the absorption spectrum of the solution was recorded in the range 550-800 nm.

In addition to the generally accepted calculation of the potential using the Nernst equation, the potential was measured directly by redox titration on platinum electrodes in a cell for bioelectrochemical studies with simultaneous spectrophotometric assay of the sample. The cell construction and the measurement procedure were described in [16]. The potential was measured using a I-130.1 ionomer (Russia). A EVL-1MZ Ag/AgCl electrode (E = 201 ± 2 mV) (Russia) was used as the reference electrode in all measurements described.

All chemicals used were of analytical grade.

RESULTS AND DISCUSSION

The study of *C. maxima* culture growth showed that laccase activity was maximal on the 18th day of surface growth and on the 9th day of submerged growth. The enzyme activity in the culture medium was twofold higher in the case of submerged cultivation. Thus, further cultivation was performed using the submerged method.

The decrease in carbohydrate content of the nutrient medium reached 50% by the 3rd-4th day of growth, this correlating with rapid accumulation of biomass in this period (Fig. 1), while the enzyme activity remained low (18% of the maximal value). Maximal accumulation of the fungal mycelium was observed on the 9th-11th day. The pH value of the culture liquid changed from 5.8 to 3.8 by the 4th day of cultivation with subsequent increase to 4.8 (8th day) and then remained constant to the end of the cultivation (Fig. 1). Maximal activity was observed in the culture medium after the pH of the medium reached 4.8 (the optimal value for the activity of the enzyme). The maximum of fungal biomass accumulation correlated with the peak of laccase activity (9th-11th days). The dynamics of the changes in pH, activity, and fungal biomass differed from that obtained for Basidiomycetes by other authors [11, 14, 17]. In the case of submerged cultivation of the Basidiomycetes *Coriolus hirsutus*, maximal accumulation of the enzyme was observed on the 7th-8th day [13], and in the case of *Cerrena unicolor* on the 12th

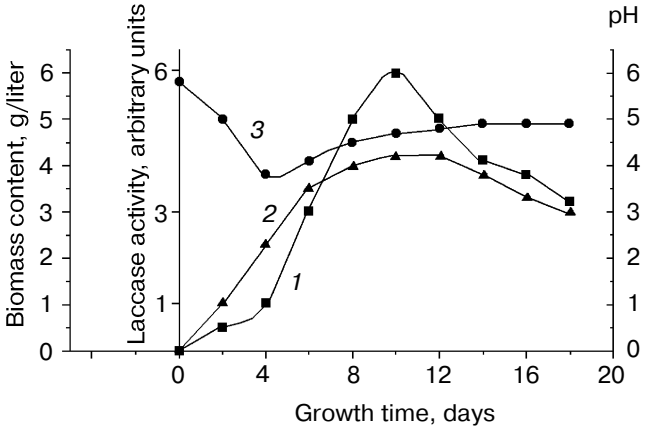

Fig. 1. Dependence of extracellular laccase activity on the cultivation time for submerged growth of *C. maxima*: *1*) laccase activity; *2*) mycelium accumulation; *3*) pH.

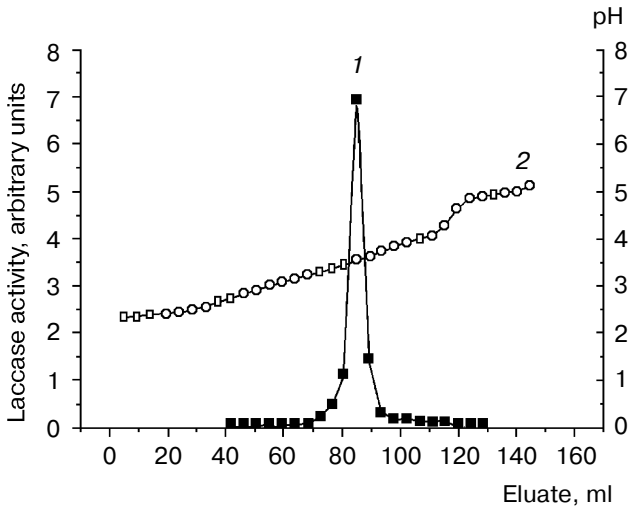

Fig. 2. Isoelectric focusing of *C. maxima* culture liquid: *1*) laccase activity; *2*) pH. Measurement error for activity assay is 0.3%.

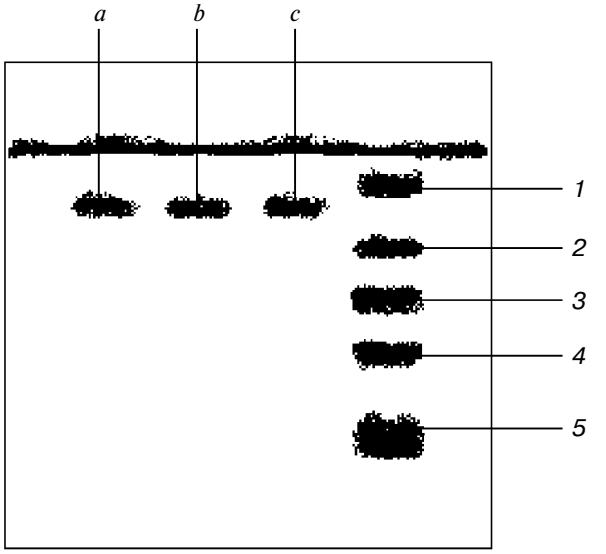

Fig. 3. SDS-PAGE of extracellular laccase isoforms from the Basidiomycetes *C. maxima*: a) 1st isoform (p*I* 3.53); b) 2nd isoform (p*I* 3.91); c) 3rd isoform (p*I* 4.25). Protein markers: *1*) albumin (67 kD); *2*) ovalbumin (43 kD); *3*) carboanhydrase (30 kD); *4*) trypsin inhibitor (20.1 kD); *5*) α-lactalbumin (14.4 kD).

day [19]. For both *C. hirsutus* and *C. unicolor* the activity was maximal when the pH of culture liquid decreased; a subsequent increase in pH caused a sharp drop in the enzyme activity in the medium.

Laccase and Mn-peroxidase isoenzymes were separated by isoelectric focusing. Three laccase isoforms were observed with p*I* 3.53, 3.91, and 4.25, respectively (Fig. 2). The contents of the second and third isoforms were very small: their activities amounted to 3 and 2%, respectively, of the total activity. Multiple isoforms of laccases with similar p*I* values are often observed for basidium fungi [11, 17-19], and virtually all the laccases investigated have p*I* values in the acidic pH range [12, 17-20]. Further purification resulted in isolation of the homogeneous isoenzymes as shown by SDS-PAGE (Fig. 3). The molecular weight of the isoforms was 57 kD, which is in good agreement with data for other fungal laccases: *Coriolus versicolor* (62 kD) [21], *C. hirsutus* (55 kD) [11], *Coriolus zonatus* (60 kD) [17].

Since the content of two isoforms in the enzyme preparations was very small, we further investigated the main laccase isoform with p*I* 3.53. Its activity was constant at pH values in the range 3.0-5.0 (Fig. 4). According to [10], optimal pH values for oxidation of phenolic substrates are close to p*I* values of laccases of the arboreal fungi *Coriolus subvermisporo* (p*I* 3.46-3.63, pH optimum 4.82) [18], *C. hirsutus* (p*I* 4, pH optimum 4-4.5) [11], and *C. zonatus* (p*I* 4.6, pH optimum 4.5-4.9) [17].

The catalytic activity of the enzyme is maximal at 50°C (Fig. 5).

The stability of the laccase was investigated in 0.1 M acetate buffer (pH 4.5) at 40°C (Fig. 6). After 27 h of

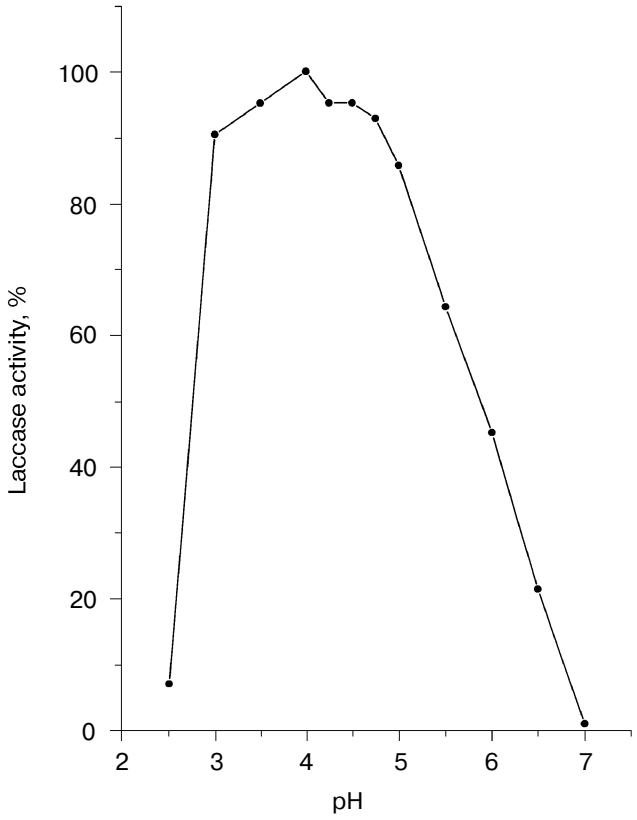

Fig. 4. pH dependence of laccase activity. The activity at pH 4.25 was taken as 100%.

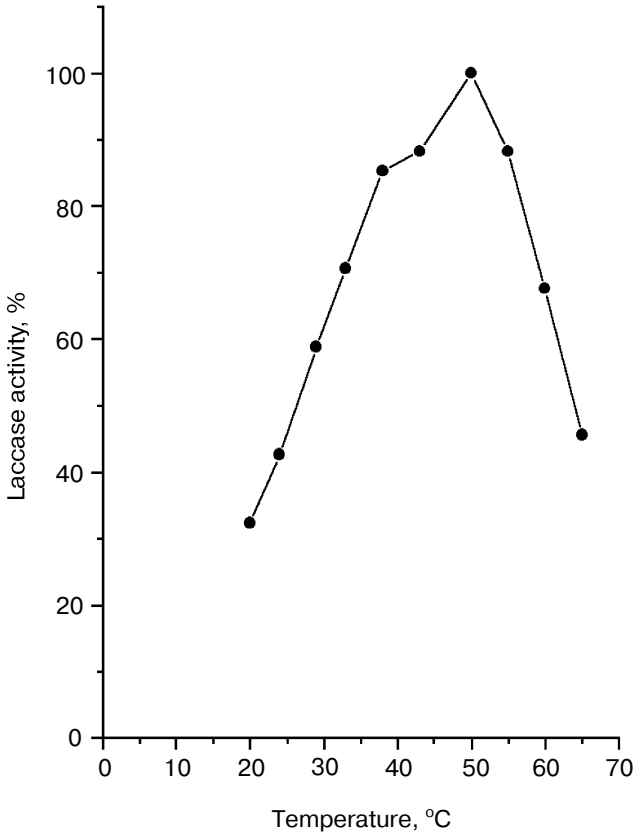

Fig. 5. Temperature dependence of laccase activity. The activity at 50°C was taken as 100%.

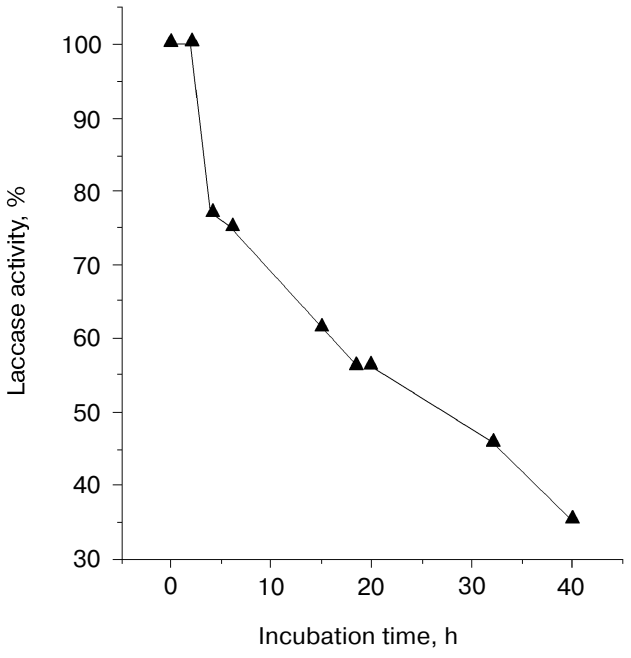

Fig. 6. Inactivation of laccase from the Basidiomycetes *C. maxima* at 40°C.

Kinetic parameters for the laccase from *C. maxima*

Substrate	K_m, μM	k_{cat}, sec^{-1}
Catechol	122	675
Hydroquinone	95	625
Sinapinic acid	23	652
$K_4[Fe(CN)_6]$	115	930

incubation, the enzyme lost about 50% of its activity. Thus, this laccase has high stability, comparable with that of other fungal laccases. For example, the half-inactivation time for laccase from *C. zonatus* was 27 h [17], and for laccase from *C. hirsutus* it was 43 h under the same conditions [11].

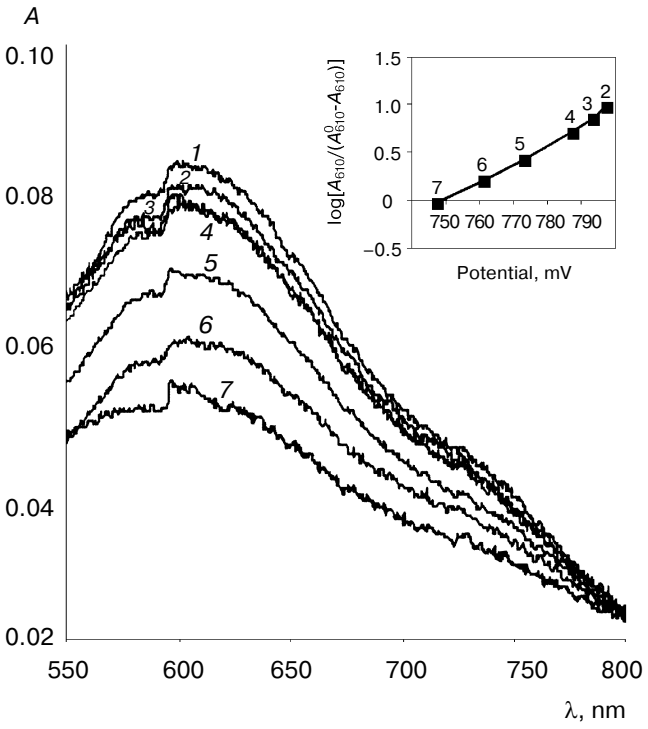

Fig. 7. Potentiometric titration of the *C. maxima* laccase T1 site under anaerobic conditions. A^0_{610} and A_{610}, optical density (at 610 nm) of the oxidized and partly reduced laccase T1 site, respectively. *1*) Optical absorption spectrum for T1 site of native enzyme (42 μM); *2*) the enzyme in the presence of 0.5 mM $K_3Mo(CN)_8$ and 0.1 mM $K_4Mo(CN)_8$; *3*) the enzyme in the presence of 0.5 mM $K_3Mo(CN)_8$ and 0.2 mM $K_4Mo(CN)_8$; *4*) the enzyme in the presence of 0.5 mM $K_3Mo(CN)_8$ and 0.3 mM $K_4Mo(CN)_8$; *5*) the enzyme in the presence of 0.5 mM $K_3Mo(CN)_8$ and 0.5 mM $K_4Mo(CN)_8$; *6*) the enzyme in the presence of 0.5 mM $K_3Mo(CN)_8$ and 1 mM $K_4Mo(CN)_8$; *7*) the enzyme in the presence of 0.5 mM $K_3Mo(CN)_8$ and 2 mM $K_4Mo(CN)_8$.

The laccase from *C. maxima* was titrated using the potassium ferri/ferrocyanide redox couple. Even at $K_3Fe(CN)_6/K_4Fe(CN)_6$ ratio 100 (E = 0.55 V), virtually complete reduction of the T1 site was achieved (detected by the disappearance of the absorption band at 610 nm). This indicates a high E^o value of the enzyme (exceeding 550 mV) and suggests using mediators with higher E^o value for measuring the laccase potential.

The K_m and k_{cat} values for four various substrates are presented in the table. It is noteworthy that the values of the constants are higher than those obtained for homogeneous laccase preparations from the fungi *C. hirsutus* [11] and *C. zonatus* [17]. This suggests that the enzyme has a rather high catalytic ability for oxidizing both phenolic compounds and inorganic substrates.

The standard redox potential of the *C. maxima* laccase T1 site (E° = 750 ± 5 mV) was determined using $K_3Mo(CN)_8/K_4Mo(CN)_8$ as the redox couple. The measured E^o for the $K_3Mo(CN)_8/K_4Mo(CN)_8$ couple was 778 mV, which is in agreement with the literature data [10]. The optical density of $K_3Mo(CN)_8$ and $K_4Mo(CN)_8$ absorption spectra in the range of 610 nm is close to zero, this allowing the laccase T1 sites to be spectrophotometrically detected with high accuracy.

Figure 7 shows the changes in laccase absorption spectrum during the reduction of the T1 site. The potential was calculated according to the equation: $E = E^\circ + 0.059 \log\{[K_3Mo(CN)_8]/[K_4Mo(CN)_8]\}$, where E° = 0.778 V. Equilibrium concentrations of $K_3Mo(CN)_8/K_4Mo(CN)_8$ were calculated considering the amount of the reduced enzyme. The potential measured by the direct potentiometric titration with platinum electrodes was close to the value obtained by the theoretical calculation, indicating the validity of the result. The redox reaction of the T1 site involves a single electron, since the slope of the Tale plot is 56 mV (see insert to Fig. 7).

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