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# Comparative Characterization of Methods for Removal of Cu(II) from the Active Sites of Fungal Laccases

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**Abstract**—Copper-containing sites of laccases isolated from the Basidiomycetes *Coriolus hirsutus* and *Coriolus zonatus* were characterized by optical methods and EPR spectroscopy. Methods for preparation of fungal laccase derivatives free from type 2 copper ions were compared. The data of EPR spectroscopy and spectrophotometric titration of copper sites showed that only a modified method based on the use of bathocuproine as a chelator for type 2 copper yielded laccase derivatives completely free from type 2 copper. The original enzymes can be reconstituted from the derivatives by dialysis under anaerobic conditions, resulting in complete recovery of native conformation of the protein molecule and the structure of the copper-containing site.

*Key words*: laccase, copper sites, EPR spectroscopy, removal of type 2 copper, bathocuproine, reconstitution of laccase

Copper-containing oxidases are a family of enzymes having pronounced spectral characteristics and similar primary structure; they exhibit wide substrate specificity [1]. The family is represented by laccase, ascorbate oxidase, and ceruloplasmin. Detailed study of these enzymes has revealed copper ions in the active site structure, and these have been subdivided into three main types [2]: the first type, or "blue copper" (T1), the second type (T2), and the third type (T3), or binuclear copper site. Type 1 is characterized by an absorption maximum at 610 nm with $\varepsilon \sim 5000\ M^{-1}\cdot cm^{-1}$ and hyperfine EPR spectrum with constant $A_{\parallel}$ $(43\text{-}95)\cdot 10^{-4}\ cm^{-1}$ [3]. Type 2 exhibits characteristics of bivalent copper with $A_{\parallel}$ $(158\text{-}201)\cdot 10^{-4}\ cm^{-1}$ and has no absorption band in optical spectra [3]. Type 3, or binuclear copper complex, makes a contribution in the enzyme spectrum by a shoulder of the absorption peak at 330 nm ($\varepsilon \sim 5000\ M^{-1}\cdot cm^{-1}$) and cannot be revealed by EPR spectroscopy because of strong anti-ferromagnetic coupling [3]. The sites T2 and T3 form a tri-nuclear cluster that has been demonstrated by spectral and X-ray diffraction studies [4].

Laccase (monophenol, dihydroxyphenylalanine: oxygen-oxidoreductase, EC 1.14.18.1) is one of the simplest representatives of this family; it contains four copper ions of the three types described. However, study of the active site structure and the mechanism of the enzyme is hindered by lack of information on its crystal structure [1-3].

One of the main approaches to the study of the active site structure of laccase is investigation of its T2-depleted derivatives [5], or derivatives containing various substitute metals at the T1 site [6]. For laccase from the fungus *Coriolus versicolor*, T2 was shown to be selectively removed by dialysis against buffer containing cyanide at pH 7.0 [7]. However, for the plant laccase from *Rhus vernicifera*, this method resulted in the removal of all copper ions, and in spite of varying the experimental conditions, selective removal of T2 was not achieved [8]. Several authors have described the possibility of using of chelators—dimethylglyoxime [9], diethyldithiocarbamate, and bathocuproine [7, 9, 10]—to achieve selective T2 removal from laccase *R. vernicifera* under reducing conditions. However, these procedures yielded at least three different forms of laccase [11]. Recently, a new approach was developed to obtain T2-depleted (T2D) laccase from *R. vernicifera* based on the use of redox buffer containing ferro- and ferricyanide in the presence of EDTA [12]. The procedure resulted in preparing only the enzyme form free from type 2 copper ion. Data on preparation of fungal T2D-laccases are virtually absent. Thus, developing methods for T2 removal from the active site of fungal laccases for

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the investigation of the mechanisms of their functioning and electron transfer processes in the course of catalysis is a subject of current interest.

In the course of our previous studies, two strains of basidial fungi producing extracellular laccase were found—*Coriolus hirsutus* and *Coriolus zonatus* [13, 14], and the basic chemical and physical properties of these enzymes were described.

The aim of the present work was to compare various methods for T2 removal from the active site of fungal laccases for subsequent investigation of their active site structure and mechanism of action.

## MATERIALS AND METHODS

**Strains** producing extracellular laccases were obtained from the Komarov Botanical Institute, Russian Academy of Sciences (St. Petersburg). The strains were cultivated as previously described [13, 14].

Homogeneous preparations of extracellular laccases from the Basidiomycetes *C. hirsutus and C. zonatus* were obtained using the procedures described in [13, 14]. Homogeneity of the preparations was checked by HPLC using an FPLC unit from Pharmacia (Sweden) and gel-filtration column TSK 3000. The proteins were eluted with 50 mM potassium phosphate buffer, pH 6.5, at 0.5 ml/h.

**Laccase activity** was detected spectrophotometrically [15] at 410 nm (Hitachi-557, Japan) using 10 mM catechol (Sigma, USA) as chromogenic substrate; the reaction was performed in 0.1 M sodium acetate buffer, pH 4.9. The activity was expressed as the amount of the enzyme that caused an increase in absorbance of 0.1 per min in 1 ml of the reaction mixture.

Protein concentration was determined by the method of Lowry et al.

**Copper amount** was determined spectrophotometrically using 2,2′-biquinoline (Merck, Germany) [16].

**Optical spectra** of the laccases were investigated in 50 mM potassium phosphate buffer, pH 6.0, in a 1 cm cuvette at 25°C.

**EPR spectra** were recorded on an RE-1306 radio spectrometer (Chernogolovka, Russia) at 9.4 Hz and 77 K. All the spectra were recorded in 50 mM potassium phosphate buffer, pH 6.0. Samples for EPR spectroscopy were prepared by quick freezing of the solutions in liquid nitrogen. The EPR spectra were recorded at 20 mW; scanning in the range 5-50 mW did not significantly alter the protein spectra. Therefore, the amplitude modulation used in the experiments was 1.0-2.7 G. To calibrate field scanning and to measure *g*-factors, $Mn^{2+}$ in MnO were used as standards. EPR spectra were simulated using a program developed in FORTRAN 77 for an axial anisotropy model [17]. Zeeman and hyperfine interactions were only considered in the spin Hamiltonian. Spectral shape was computed assuming the angular dependence of the transition probability [18] and using a Lorenz curve for each spectral line.

**CD spectra** were recorded on a JASCO-40AS spectrometer (Japan) in 1-2-mm cuvettes at 195-250 nm; the experiments were performed at room temperature.

**Removal of T2 from laccase using redox buffer and subsequent reconstitution of the enzyme.** Laccase preparations were dialyzed against deionized water and concentrated by ultrafiltration to final concentration 20-25 mg/ml. Then T2 was removed by dialysis against 50 mM sodium acetate buffer, pH 5.2, containing 5.4 mM potassium ferrocyanide (Merck), 0.6 mM potassium ferricyanide (Merck), and 5 mM EDTA (Khimmed, Russia) for 6-8 h. Unreacted compounds were removed by dialysis against 15 mM potassium phosphate buffer, pH 6.0. The resulting preparation was applied onto a column (1 × 7 cm) filled with DEAE-Toyopearl 650 M (Toyo-Soda, Japan) and eluted with 15-100 mM gradient of potassium phosphate buffer, pH 6.0. Copper incorporation into T2D laccases was performed under anaerobic conditions by dialysis against 50 mM sodium acetate buffer, pH 5.2, in the presence of 28 mM ascorbic acid and 2-3 mg-eq of Cu(I) or Cu(II) at 5°C for 3-4 h. Then the preparation was dialyzed against 50 mM sodium acetate buffer, pH 5.2, for 2 days with repeated changes of the buffer.

**Removal of T2 from laccase using bathocuproine and subsequent reconstitution of the enzyme.** The reaction mixture (final volume 6 ml) contained 0.13 mM laccase, 28 mM ascorbic acid, 1 M guanidine hydrochloride (Merck), and 1.2 mM bathocuproine disulfonate (Merck) in 100 mM sodium acetate buffer, pH 4.5. The reaction was initiated by addition of bathocuproine solution, and after various time intervals was stopped by cooling in an ice bath. The enzyme was separated by gel-filtration on Sephadex G-25 column (2 × 55 cm) (Pharmacia) equilibrated by 20 mM potassium phosphate buffer, pH 6.0, at 4°C. Fractions containing laccase were pooled and concentrated by ultrafiltration. Copper was incorporated into T2D laccase under anaerobic conditions by dialysis against 100 mM sodium citrate buffer, pH 6.0, containing 28 mM ascorbic acid and 50 μM Cu(II) for 3-4 h at room temperature; then the preparation was dialyzed against 25 mM sodium phosphate buffer, pH 6.0, in the presence of 1 mM EDTA for 20 h at 4°C with double change of the buffer. Finally, the sample was dialyzed against 25 mM sodium phosphate buffer, pH 6.0, for 12 h at 4°C with threefold change of the buffer.

To avoid traces of metals, all buffer solutions were prepared using deionized water and then passed through a Chelex-100 column (BioRad, USA). Glassware was left in concentrated HCl for at least 12 h. Anaerobic conditions were maintained by saturation of the solutions with nitrogen.

All inorganic chemicals were of analytical grade.

## RESULTS AND DISCUSSION

**Characterization of copper sites in native fungal laccases.** Laccase preparations from *C. zonatus* and *C. hirsutus* were homogeneous by SDS-PAGE and HLPC analysis and contained four copper ions, this being consistent with data on other laccases [1, 3, 19]. The optical spectra of the laccases were very similar to the spectrum of laccase from *C. versicolor*, exhibiting absorption bands at 605-610 nm and 280 nm and a shoulder at 330 nm [1, 20] (Fig. 1). Thus, the laccases contained type 1 copper ion (the band at 605-610 nm) as well as two ions of type 3 copper, which form a binuclear complex (the shoulder at 330 nm). The presence of type 2 copper was confirmed by EPR investigations of the native enzymes. EPR spectra of both laccases were very similar to the spectrum of the laccase from *R. vernicifera* [21] (Fig. 2), this arising from the combination of EPR signals from type 1 and type 2 copper sites (spectra *1a*, *3a*, and *1b*, *3b*). To determine parameters of *A*- and *g*-tensors, computer simulation of the experimental spectra was performed. Since the spectra are combined from two signals (from type 1 and type 2 copper ions), we used the following approach: using the experimental values of tensors $g_{\|}$, $A_{\|}$, $\Delta H$ and approximate values of parameters of $g_{\perp}$, EPR spectra were simulated for type 1 and type 2 copper ions separately. Then the sum of the theoretical spectra obtained (type 1 and type 2 copper) was compared with the experimental spectrum, and the best fit was achieved by concerted changing

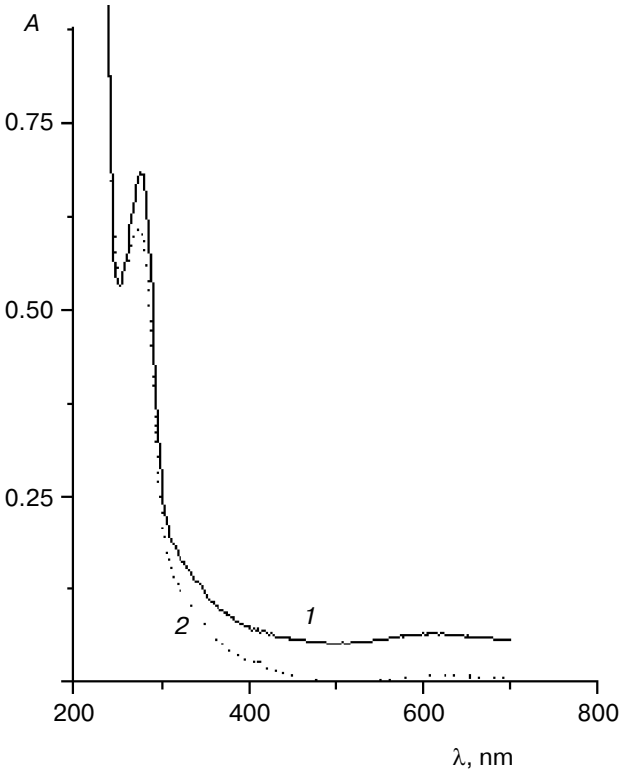


**Fig. 1.** Vertically shifted optical spectra of laccases from *C. hirsutus* (*1*) and *C. zonatus* (*2*). The spectra were recorded in 50 mM potassium phosphate buffer, pH 6.0, 25°C.

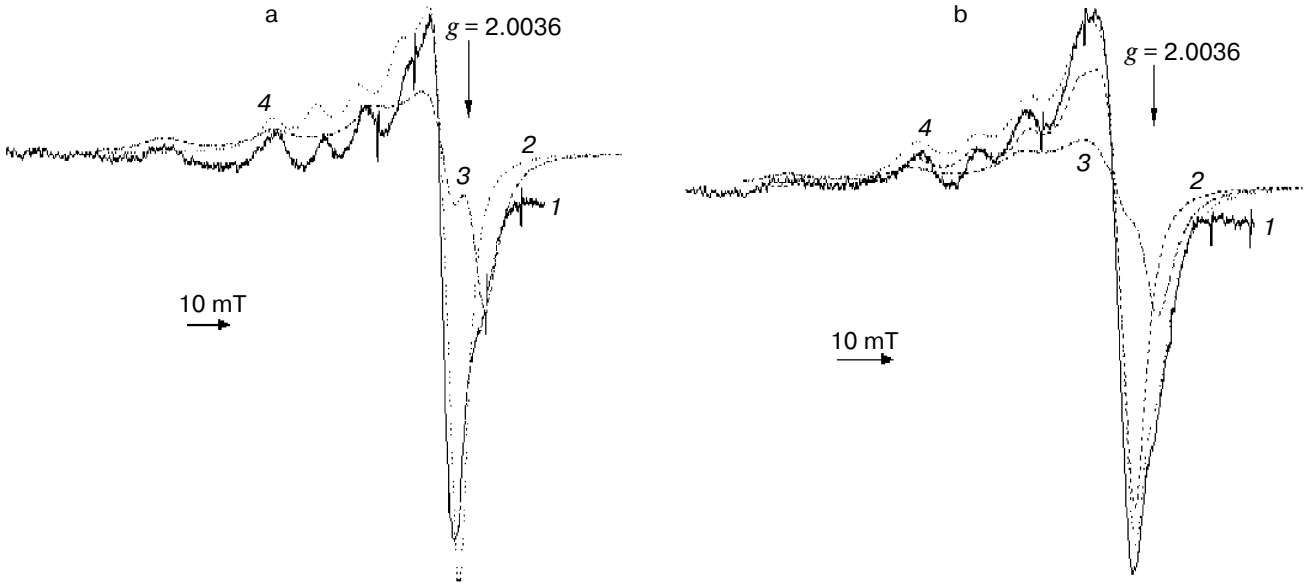


**Fig. 2.** Experimental and simulated EPR spectra of laccases from *C. hirsutus* (a) and *C. zonatus* (b): *1*) experimental spectra; *2*) simulated spectra for T1; *3*) simulated spectra for T2; *4*) sum of the simulated spectra *2* and *3*.

**Table 1.** Calculated parameters of *A*- and *g*-tensors and $\Delta H$ for laccases from *C. hirsutus* and *C. zonatus*

| Laccase from | Copper type | $g_{\parallel}$ | $g_{\perp}$ | $A_{\parallel}$, G | $A_{\perp}$*, G | $\Delta H$, G |
|---|---|---|---|---|---|---|
| *C. hirsutus* | I | 2.185 | 2.030 | 80 | 15 | 25 |
| | II | 2.205 | 2.033 | 180 | 14 | 40 |
| *C. zonatus* | I | 2.203 | 2.040 | 95 | 15 | 30 |
| | II | 2.250 | 2.042 | 190 | 17 | 45 |

* Since $A_{\perp}$ cannot be observed in the experimental spectra because of the greater value of $\Delta H$, the values calculated are approximate.

of spin-Hamiltonian parameters in the computer-simulated spectra. The parameters of *A*- and *g*-tensors for the experimental spectrum were determined (Table 1). These values are close to those for other laccases [3, 21].

Titration with hydrogen peroxide [12] oxidized type 1 copper ions by 75-80% (mean of 5 experiments), and type 3 copper ions (T3) were completely oxidized. The preparations of the native laccases were used to obtain T2-depleted enzyme derivatives.

**Preparation of T2-depleted laccase derivatives.** The method based on the use of redox buffer containing EDTA was only effective for the laccase from *C. hirsutus*. In the case of the laccase from *C. zonatus*, we failed to remove type 2 copper ions using this approach, and even varying the ratio of potassium ferrocyanide (0-6 mM) and potassium ferricyanide (0-6 mM), or increase of the reaction time to 16 h had no result. No changes in EPR spectra, enzymatic activity, or total copper content in the laccase were observed on varying the parameters indicated above. We think it likely that these results are due to a significant difference in the redox potential values for the copper ions in laccase from *C. zonatus* and the laccase from *R. vernicifera* (for which the method was developed). The potential values for type 2 copper ions are known only for the laccase from *R. vernicifera* [22], and this hinders the interpretation of these data.

For laccase from *C. hirsutus*, as well as *R. vernicifera*, the method described allows removal of ~1 copper ion per enzyme molecule (Table 2). However, we did not observe enzyme denaturation in our experiments—the T2D laccase derivative was eluted from the ion-exchange column as a single peak, and the elution profile was identical to that of the native enzyme. T2-depleted laccase retained up to 30% of its enzymatic activity, in contrast with the data of other authors [12, 23] on the complete, or virtually complete (by 90%) inactivation of the enzyme. Although the analysis of EPR spectra of laccases free from copper ions showed some change in spectral shape due to a decrease in EPR signal from type 2 copper, hyperfine structure components from T2 were observed in these spectra, indicating incomplete T2 removal.

**Table 2**. Characterization of laccase from *C. hirsutus* and its T2-depleted derivatives prepared using redox buffer

| Enzyme preparation | Number of copper atoms per laccase molecule | Relative activity*, % |
|---|---|---|
| Native laccase | 4.1 ± 0.15 | 100 |
| T2-depleted laccase | 3.0 ± 0.15 | 33 |
| Reconstituted laccase ($Cu^{2+}$ with ascorbic acid) | 4.0 ± 0.15 | 108 |
| Reconstituted laccase ($Cu^{+}$ with ascorbic acid) | 3.9 ± 0.15 | 88 |

* Error did not exceed 10%.

Study of the interaction of *C. hirsutus* and *C. zonatus* laccases with bathocuproine demonstrated that the native enzymes as well as the completely reconstituted enzymes did not react with this chelator, a necessary condition for the reaction being the presence of guanidine hydrochloride (a denaturing agent) in the reaction mixture. In the presence of guanidine hydrochloride, copper ions bind to bathocuproine yielding a complex that exhibits a specific absorption band at 480 nm (Fig. 3). The reaction with bathocuproine strongly depends on the pH of the reaction mixture: only 10-15% of the copper can be removed from the *C. hirsutus* laccase in 40 min and from the *C. zonatus* laccase in 60 min of incubation at pH 5.2. At pH 4.0, 25% of total copper was removed from both laccases in the same time of incubation. Increasing the incubation time to 4 h at pH 5.2 or 4.0 did not lead to an increase in absorption at 480 nm. This indicated no further chelation of copper with bathocuproine. The fungal laccase preparations lost ~85-90% of their activity and 25-30% of their total copper after the reaction with bathocuproine. The results are summarized in Table 3. According to EPR data, the copper removed by this method of type 2 (Fig. 4).

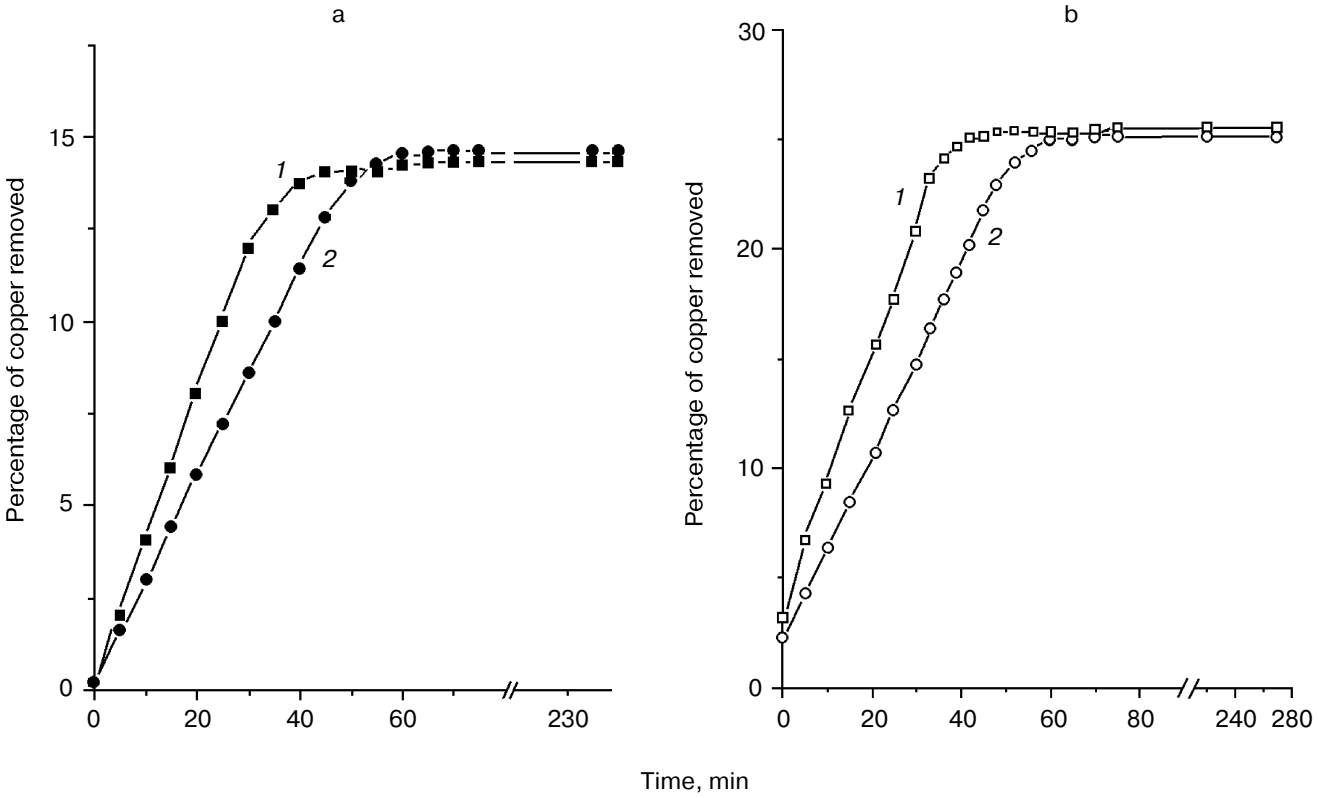


**Fig. 3.** pH dependence of the amount of copper removed from laccases from *C. hirsutus* (*1*) and *C. zonatus* (*2*) at 20°C. The reaction mixture contained 1 M guanidine hydrochloride, 28 mM ascorbic acid, 0.13 mM laccase, and 1.2 mM bathocuproine disulfonate in 0.1 M acetate buffer, pH 5.2 (a) and pH 4.0 (b).

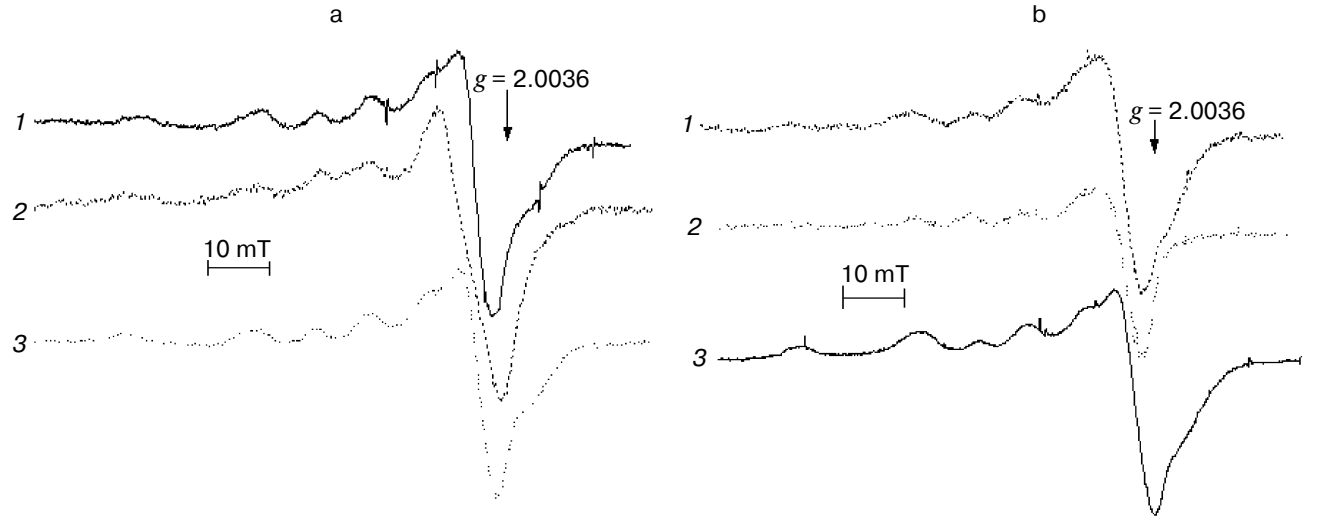


**Fig. 4.** Experimental EPR spectra of laccases from *C. hirsutus* (a) and *C. zonatus* (b) and their derivatives: *1*) native enzyme; *2*) type 2 copper depleted derivatives; *3*) reconstructed enzyme.

Ion-exchange chromatography of these derivatives resulted in single peaks, and the elution profiles and elution volumes did not differ from those of the native enzymes. Comparing the CD spectra of native enzymes and their T2D derivatives did not reveal any difference at 215 nm, which indicates native conformation of the enzymes (Fig. 5). Although type 1 copper is very sensitive to enzyme denaturation [2, 3, 24], EPR spectra of T2-

**Table 3**. Characterization of laccases from *C. zonatus* and *C. hirsutus* and their derivatives liberated from type 2 copper centers using bathocuproine

| Enzyme preparation | *C. zonatus* | | *C. hirsutus* | |
|---|---|---|---|---|
| | number of copper atoms per laccase molecule | relative activity*, % | number of copper atoms per laccase molecule | relative activity*, % |
| Native laccase | 3.9 ± 0.15 | 100 | 4.1 ± 0.15 | 100 |
| Type 2 copper-depleted laccase | 2.7 ± 0.15 | 14 | 2.9 ± 0.15 | 16 |
| Reconstituted laccase ($Cu^{2+}$ with ascorbic acid) | 4.0 ± 0.15 | 105 | 4.0 ± 0.15 | 110 |
| Reconstituted laccase ($Cu^{+}$ with ascorbic acid) | 4.0 ± 0.15 | 82 | 3.9 ± 0.15 | 73 |

* Error did not exceed 10%.

depleted laccase derivatives did not reveal any changes in the environment of type 1 copper. Optical studies also did not reveal any change in either optical spectra or molar absorption coefficient of this copper type after the removal of T2 copper. These results revealed no denaturation in the protein part of the laccase molecules.

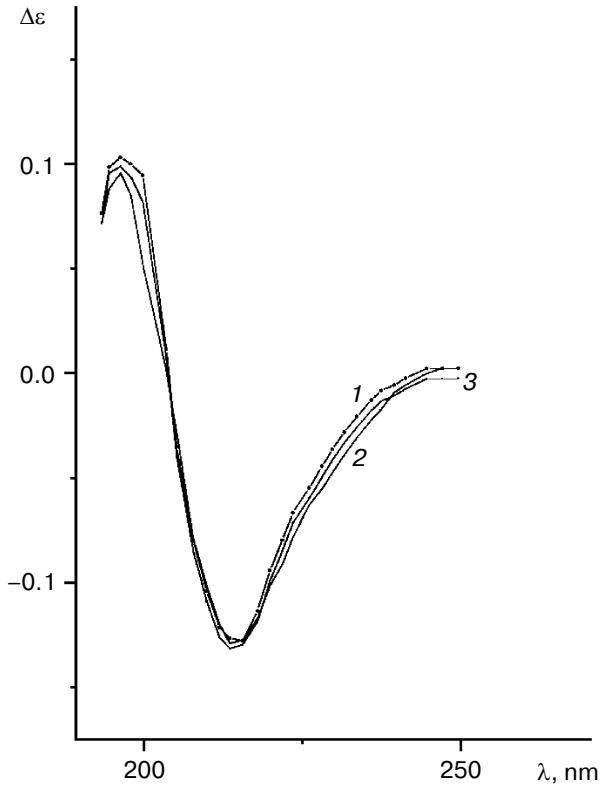


**Fig. 5.** CD spectra of laccase from *C. hirsutus* (*1*), type 2 copper depleted derivative of the enzyme (*2*), and the reconstituted enzyme (*3*). The spectra were recorded in 1-mm cuvette in 50 mM potassium phosphate buffer, pH 6.0, at 25°C.

As previously shown for plant laccases, the environment of type 1 copper ion can be accurately determined in the enzyme free from type 2 copper ions [3, 25]. This was confirmed in our experiments. These laccase derivatives exhibited pronounced square configuration of nitrogen atoms surrounding type 1 copper in the equatorial plane and had a parameter of the additional hyperfine splitting from four equivalent nitrogen atoms $A_N$ = 10.4 gauss for both fungal laccases.

**Reconstitution of type 2 copper-depleted laccase derivatives.** Dialysis of T2-depleted laccases against buffer solutions containing monovalent or bivalent copper in the presence of ascorbic acid resulted in complete reactivation of the enzyme (Tables 2 and 3). Reconstituted preparations contained 3.9-4.0 copper atoms, and the shapes of EPR spectra and parameters of the spin Hamiltonian completely corresponded to the native form of the enzyme (Fig. 4). These indicate the identity of the ligand surrounding of T2 copper in the native and reconstituted laccases, and, consequently, the structural recovery of the enzyme sites containing type 2 copper ions.

Thus, comparison of two methods for removal of type 2 copper demonstrated the suitability of bathocuproine for preparing of T2-depleted derivatives of both fungal laccases. This method does not cause serious changes either in the protein part of the enzyme molecule or in the surrounding of the type 1 copper ions and allows complete reconstitution of the enzyme. The method using redox buffers in the presence of EDTA is not of general purpose, and its applicability depends on various electrochemical parameters of copper ions; moreover, it did not allow complete removal of type 2 copper ion from the protein molecules of the laccase from *C. hirsutus*.

## REFERENCES


1. Yaropolov, A. I., Skorobogat'ko, O. V., Vartanov, S. S., and Varfolomeyev, S. D. (1994) *Appl. Biochem. Biotechnol.*, **49**, 257-275.
2. Peter, M. G., and Wollenberger, U. (1997) in *Frontiers in Biosensors. Fundamental Aspects* (Scheller, F. W., Shubert, F., and Fedrowitz, J., eds.) Birkhauser Verlag, Basel, pp. 63-82.
3. Solomon, E. I., Sundaram, U. M., and Machonkin, T. E. (1996) *Chem. Rev.*, **96**, 2563-2605.
4. Cole, J. L., Tan, G. O., Yang, E. K., Hogson, K. O., and Solomon, E. I. (1990) *J. Am. Chem. Soc.*, **112**, 2248-2249.
5. McMillin, R., and Eggleston, M. K. (1997) in *Multicopper Oxidases* (Messerschmidt, A., ed.) World Scientific, Singapore-New Jersey-London-Hong Kong, pp. 129-166.
6. Li, J.-B., and McMillin, D. R. (1990) *Inorg. Chim. Acta*, **167**, 119-122.
7. Hanna, P. M., McMillin, D. R., Pasenkiewicz-Gierula, M., Antholine, W. E., and Reinhammar, B. (1988) *Biochem. J.*, **253**, 561-568.
8. Morie-Bebel, M. M., Morris, M. C., Menzie, J. L., and McMillin, D. R. (1984) *J. Am. Chem. Soc.*, **106**, 3677-3678.
9. Graziani, M. T., Morpurgo, L., Rotilio, G., and Mondovi, B. (1976) *FEBS Lett.*, **70**, 87-90.
10. Morpurgo, L., Desideri, A., Rotilio, G., and Mondovi, B. (1980) *FEBS Lett.*, **113**, 153-156.
11. Frank, P., and Pecht, I. (1986) *J. Phys. Chem.*, **90**, 3809-3814.
12. Klemens, A. S., and McMillin, D. R. (1990) *J. Inorg. Biochem.*, **38**, 107-115.
13. Koroleva-Skorobogat'ko, O., Stepanova, E., Gavrilova, V., Morozova, O., Lubimova, N., Dzchafarova, A., Yaropolov, A., and Makower, A. (1988) *J. Biotechnol. Appl. Biochem.*, **28**, 47-54.
14. Koroleva (Skorobogat'ko), O., Stepanova, E., Gavrilova, V., Biniukov, V., Jaropolov, A., Varfolomeyev, S., Scheller, F., Makower, A., and Otto, A. (1999) *Appl. Biochem. Biotechnol.*, **76**, 115-118.
15. Varfolomeyev, S., Naki, A., and Yaropolov, A. I. (1985) *Biokhimiya*, **50**, 1411-1419.
16. Malkin, R., Malmstrom, B. G., and Vanngard, T. (1969) *Eur. J. Biochem.*, **7**, 253-259.
17. Huang, H.-W., Sakurai, T., Maritabo, S., Marchesini, A., and Suzuki, Sh. (1999) *J. Inorg. Biochem.*, **75**, 20-25.
18. Abragam, A., and Bleany, B. (1970) *Electron Paramagnetic Resonance of Transition Ions*, Clarendon Press, Oxford.
19. Gianfreda, L., Sannino, F., Filazzola, M. T., and Leonowicz, A. (1998) *J. Mol. Catalysis B: Enzymatic*, **4**, 13-23.
20. Reinhammar, B. (1998) in *Multicopper Oxidases* (Messerschmidt, A., ed.) World Scientific, Singapore-New Jersey-London-Hong Kong, pp. 167-200.
21. Agostinelli, E., Cervoni, L., Giartosio, A., and Morpurgo, L. (1995) *Biochem. J.*, **306**, 697-702.
22. Reinhammer, B. (1978) *Biochim. Biophys. Acta*, **534**, 7-14.
23. Farver, O., Goldberg, M., Lancet, D., and Pecht, I. (1976) *Biochem. Biophys. Res. Commun.*, **73**, 494-500.
24. Naki, A., and Varfolomeev, S. D. (1980) *FEBS Lett.*, **113**, 157-160.
25. Malmstrom, B. G., Reinhammar, B., and Vanngard, T. (1968) *Biochim. Biophys. Acta*, **156**, 67-76.