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# Effect of Solvent Phase Transitions on Enzymatic Activity and Structure of Laccase from *Coriolus hirsutus*

**E. V. Stepanova[1], T. V. Fedorova[1], O. N. Sorokina[2], V. V. Volkov[3], O. V. Koroleva[1]*, and A. T. Dembo[3#]**

[1]*Bach Institute of Biochemistry, Russian Academy of Sciences, Leninsky pr. 33, 119071 Moscow, Russia; fax: (495) 954-2732; E-mail: koroleva@inbi.ras.ru*
[2]*Emmanuel Institute of Biochemical Physics, Russian Academy of Sciences, ul. Kosygina 4, 119334 Moscow, Russia; fax: (495) 137-4101; E-mail: alsiona1983@mail.ru*
[3]*Shubnikov Institute of Crystallography, Russian Academy of Sciences, Leninsky pr. 59, 119333 Moscow, Russia; fax: (495) 135-1011; E-mail: vvo@ns.crys.ras.ru*

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**Abstract**—The effect of solvent phase transitions on catalytic activity and structure of the active site of laccase produced by the Basidiomycetes *Coriolus hirsutus* 072 was studied. As shown by small-angle X-ray scattering, laccase exists in solution as a mixture of monomeric and aggregated particles in the percent ratio 85 : 15. This ratio did not change on phase transitions. A complex nature of laccase activity dynamics during thawing and further heating to 20°C was shown. Spontaneous oxidation of T1 copper center in the temperature range 12-20°C was not observed. According to spectral data, the structure of laccase active sites including all copper centers of types T1, T2, and T3 changes during the phase transition.

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*Key words*: laccase, phase transitions, small angle X-ray scattering (SAXS), EPR spectra, active site

Laccase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) belongs to the group of copper-containing blue oxidases and catalyses oxidation of a wide variety of aromatic compounds (including phenol derivatives) and inorganic substrates; oxidation is accompanied by reduction of molecular dioxygen to water [1, 2]. In contrast to plant laccases, enzymes produced by fungi and bacteria can have monomeric as well as di- and tetrameric structure. However, all of them are glycoproteins, and the content of the carbohydrate part can significantly vary qualitatively and quantitatively [3, 4]. Four copper ions and their amino acid ligands are constituents of the active site, the most conservative part of laccase. Copper ions and ligands together form a unique structure that defines the spectral and catalytic properties of the enzyme [5]. It should be noted that the architecture of copper centers changed negligibly in the course of evolution of copper-containing proteins [6]; this allows consideration of the active site of laccase as a model system for studying the properties of such copper-containing oxidases as ascorbate oxidase and ceruloplasmin. In biological systems, copper ions are classified under three types according to their optical and magnetic properties (optical absorption and EPR spectra). Type 1 (T1) copper has deep blue color due to an absorption maximum at 600 nm with molar extinction coefficient ~5000 $M^{-1}\cdot cm^{-1}$ and a low value of EPR hyperfine splitting constant $A_{\|}$ = 40-95 G. Type 2 (T2) copper does not contribute into the optical spectra but has an EPR spectrum typical of bivalent copper with $A_{\|}$ = 147-187 G. Type 3 (T3) or binuclear copper complex manifests itself as a shoulder of the absorption peak at 330 nm with molar extinction coefficient ~5000 $M^{-1}\cdot cm^{-1}$ and is not detected by EPR spectroscopy because the copper ions are antiferromagnetically coupled and bonded by a ligand [1-3]. All the three types of copper ions are present in the laccase active site, but in spite of the conservative structure of the latter, the catalytic characteristics of the various laccases are markedly different. This might be caused by the value of the reduction potential of T1 copper ion [7, 8] as well as the structure of protein and carbohydrate parts of the enzyme molecule [7]. That is why a group of laccases with high reduction potential of T1 copper ion is of special interest for study.

* To whom correspondence should be addressed.
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Earlier we found strains of Basidiomycetes that are producers of laccases with high reduction potentials [9-11]. The data indicate that extracellular laccase produced by *Coriolus hirsutus* is the most promising enzyme for biotechnology. The specific feature of this enzyme is high thermal stability, most probably arising from its spatial organization and the structure of the carbohydrate part [12, 13]. The absence of standard lyophilized enzyme preparations is the main difficulty for experiments with laccase. When fungal laccases are studied, frozen enzyme preparation (stored at −18°C) is used in laboratory practice.

It is known that reversible as well as irreversible changes in the structure of an enzyme and its state in solution can occur in the course of freezing and subsequent thawing [14, 15]. As shown by EPR spectroscopy, conformation changes occur in the structure of the active sites of laccase, ascorbate oxidase, and ceruloplasmin depending on environmental temperature changes as well as in the course of freezing/thawing of protein solutions [12, 16-20]. However, the literature data are contradictory; EPR spectra are recorded under various conditions, thus making their comparison impossible. Only 30% of copper of T2 type is detected in laccase from *Rhus. vernicifera* and ascorbate oxidase from *Cucumis sativus* at room temperature. T2 copper becomes completely EPR-detected at 77 K (liquid nitrogen), the intensity of T1 copper signal being unchanged [16]. In contrast, as recently shown for laccase from *Trametes ochracea*, the intensity of T1 copper signal significantly decreases at 77 K compared with the T2 copper signal [20]. Such contradictions might be rationalized by existence of numerous intermediate forms as well as monomeric and aggregated enzyme particles in laccase solutions. This phenomenon most probably causes significant difference in catalytic parameters (see [21] as the most complete review). Accounting for the fact that for laccase the detailed catalytic mechanism is not yet elucidated, the study of the aggregation state of laccases with high reduction potential in solutions, especially at concentrations usually used for studies of catalytic mechanism, is of special interest and practical importance.

The goal of this work was to study the catalytic, spectral properties, and the structural state of laccase from *C. hirsutus* in solution at the phase transition (before freezing, at freezing, and subsequent thawing of enzyme solution) in order to elucidate processes in the active site on environmental temperature changes.

## MATERIALS AND METHODS

**Reagents.** To prepare all solutions used in this study, we used chemicals from Sigma (USA) and reagents of chemically pure and extra pure grades produced in Russia.

**Isolation and purification of laccase.** The strain producing extracellular laccase was kindly donated by the Komarov Botanical Institute of the Russian Academy of Sciences (St. Petersburg). The Basidiomycetes was cultivated according to [22].

Homogenous preparation of extracellular laccase from the Basidiomycetes *C. hirsutus* was obtained by three-stage chromatographic purification [9]. Homogeneity was checked by HPLC using a column for gel filtration with Superdex-200 (16/60) from Pharmacia (Sweden), elution with 150 mM potassium phosphate buffer, pH 6.5, rate 1.0 ml/min, and also by SDS-PAGE [23]. Polyacrylamide gel (12.6%) 8 × 6 cm with 10% SDS was used.

**Protein concentration** in solution was determined colorimetrically using a BCA kit from Pierce (USA) and BSA solution as the standard.

**Laccase activity** was detected spectrophotometrically [24] (Perkin Elmer, USA) at $\lambda = 525$ nm using 10 mM syringaldazine from Sigma in 0.1 M sodium acetate buffer, pH 4.5, as a chromogenic substrate ($\varepsilon_{525}$ = 65,000 $M^{-1} \cdot cm^{-1}$). Enzyme activity was given in international units (μmol/min per mg).

**Changes in laccase activity before freezing and after thawing.** Preparation of native laccase (immediately after chromatographic purification) with protein concentration 0.5 mg/ml was cooled to 4°C and then incubated at room temperature, where its enzyme activity was evaluated. Preparation was frozen and stored at −18°C for one month, and then enzyme activity in the preparation was again evaluated during thawing and incubation at room temperature 20 ± 2°C. In all measurements, the final protein concentration in cell was $2 \cdot 10^{-8}$ M.

**Optical absorption spectra** of laccase were recorded in 50 mM potassium phosphate buffer, pH 6.5, in a 1 cm cell in the temperature range 12-25°C with the step 2-3°C using a Kontron 930 double-beam spectrophotometer (Italy) with a HAAKE F3-C thermostat (USA) and PG-20 temperature programmer.

**EPR spectra** were recorded at room temperature and at 77 K using X-band spectrometer Bruker EMX 8.7/2. At room temperature, the enzyme solution was placed in a quartz flat cell. Microwave power was 6 mW, and modulation amplitude was 10 G. Dynamics of changes in the laccase spectrum after thawing was monitored for 3 h.

At 77 K, the enzyme solution was placed in a quartz Dewar vessel filled with liquid nitrogen. Microwave power was 2 mW, modulation amplitude was 5 G. EPR spectra of a laccase sample subjected to preliminary freezing were recorded immediately after thawing and after 100 min.

Spectra were simulated using the SpinCount software from the Hendrich Metal Protein Group; the software was kindly provided by Prof. Michael Hendrich from Carnegie Mellon University (USA).

**Small-angle X-ray scattering** patterns were studied in the laboratory of small-angle X-ray scattering of the

Institute of Crystallography of the Russian Academy of Sciences using the AMUR-K small-angle X-ray diffractometer with a linear position-sensitive detector. Detector aperture was 10 × 100 mm, linear resolution 0.3 mm, and a graphite monochromator was used. The registration system of the detector was made according to the CAMAC standard. A fine-focus X-ray tube with Cu-$K_\alpha$ radiation ($\lambda$ = 1.542 Å) was used at $U$ = 30 kV and $I$ = 30 mA. For each sample, data collection time was 2000 sec. After the first experiment, the sample was frozen. Subsequent data collections were performed immediately after thawing, after 4 h, and after a day of storage at room temperature. The enzyme concentration was 0.45 mM.

## RESULTS AND DISCUSSION

Earlier it was shown that growing on peptone-glucose medium under submerged cultivation conditions, *C. hirsutus* 072 synthesizes two isoforms of laccase (C1, C2) [25]. It should be noted that these isoforms were practically always present in the enzyme preparations at various stages of its purification with the protein ratio C1/C2 = 83 : 17 and had isoelectric points in the acidic pH region close to each other. However, in contrast to most isoenzymes synthesized by Basidiomycetes, which are white rot agents, more catalytically active C1 isoform was synthesized to a greater extent than C2. Thus, we used the former for study of the effect of phase transitions on catalytic activity of the enzyme and the structure of its active site. The purification protocol developed by us yielded laccase C1 isoenzyme preparation homogenous electrophoretically and from HPLC data.

The state of laccase in solution immediately after freezing and in the subsequent 24 h was studied by small-angle X-ray scattering. Scattering intensity curves of enzyme solution (smoothed and without collimation correction) at zero time point (before freezing, immediately after transition from solid phase to liquid, after 4 and 24 h) were obtained. The curves were almost identical (data not presented here), and this allowed us to average them into a single curve for further processing. It should be noted that a curve characterizing the enzyme state before freezing differs from others, but this difference is negligible. Inertia radius $R_g$ determined from the above-mentioned curve coincided with $R_g$ value determined from the averaged curve. The inertia radius was calculated using the Guinier equation:

$$R_g^2 = \frac{\ln I(0) - \ln I(s)}{s^2}, \qquad (1)$$

which is correct for $(sR_g) < 1.3$ [26]. Here $s$ is the modulus of scattering vector equal to $(4\pi\sin\Theta)/\lambda$, $\lambda$ is the wavelength, $\Theta$ is the half of scattering angle, $I(s)$ is experimental scattering intensity in arbitrary units. This equation

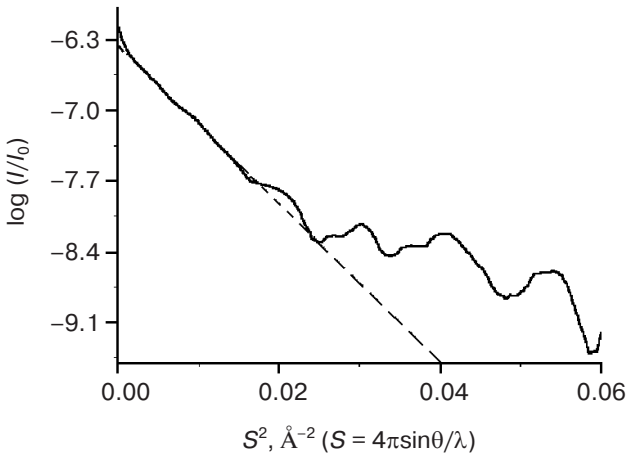


**Fig. 1.** Averaged intensity curve of small-angle X-ray scattering of laccase in Guinier coordinates after smoothing and collimation correction.

allows determination of inertia radius as a square root of the slope of the linear portion of the Guinier plot (ln $I(s)$ versus $s^2$). The averaged curve in Guinier coordinates after smoothing and collimation correction is presented in Fig. 1. A line drawn along the initial portion of the curve yields $R_g$ value of particles equal to 23.3 Å. The radius of a spherical particle with such inertia radius is $1.3 \cdot R_g$ = 30.3 Å.

To refine the dimensions of laccase particles, we plotted (Fig. 2) the distance distribution function $p(r)$:

$$p(r) = r^2\gamma(r) = \frac{1}{2\pi^2}\int_{s=0}^{\infty} I(s)\cdot\frac{\sin(sr)}{sr}ds, \qquad (2)$$

where $r$ is the length of a line segment inside the particle, and $\gamma(r)$ is the characteristic (autocorrelation) function.

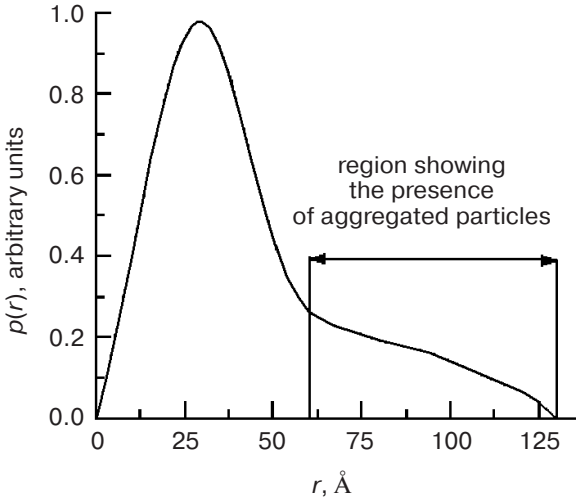


**Fig. 2.** Distance distribution function $p(r)$ calculated using the small-angle X-ray scattering curve.

The distance distribution function $p(r) = r^2\gamma(r)$ was calculated using indirect Fourier transform program GNOM [27]. Being a self-convolution of electron density function of a particle averaged over all its orientations, characteristic function $\gamma(r)$ attains zero value at distances $r$ exceeding the maximal dimension of a molecule. Thus, this maximal dimension can be evaluated using the $p(r)$ curve, namely, by finding maximum $r$ where $p(r)$ smoothly approaches zero value.

As is obvious from the plot, the maxima positions coincide and correspond with the sphere radius of 29 Å. This value approximately corresponds with a molecule with mass 50-60 kDa; this is in accordance with the molecular mass value earlier determined by SDS-PAGE and HPLC [9]. However, the presence of a shoulder in the region corresponding to particles with dimensions 60-130 Å indicates that particles with diameter up to 130 Å are present in solution. It was shown that protein aggregates with dimensions 100-180 Å are present in solutions of fungal laccases. Molecular masses of the studied enzymes were ~67 ± 2 kDa; this corresponds to particle dimension ~54 Å [20]. Unfortunately, for each laccase the authors used only one protein concentration, and these concentrations were different (9, 11, and 14 mg/ml). This makes comparison with their data difficult, because it was shown for ascorbate oxidase [28] that the number of aggregates and the extent of aggregation increase on increase of the enzyme concentration. However, the spectral data [20] were obtained immediately after thawing of enzyme preparations, and the authors failed to obtain more detailed information about laccase aggregates than the fact of their presence and approximate dimensions.

Dynamics of laccase *C. hirsutus* 072 behavior in solution at phase transitions was analyzed; it was shown that the number of particles more than 60 Å in diameter was approximately constant. Interestingly, freshly isolated homogenous enzyme (spectrum recorded before freezing) also contained aggregates and monomers in the same ratio. Fungal laccases studied earlier by us and other authors are monomers as shown by HPLC and electrophoresis under non-denaturing conditions. Nonetheless, both monomeric and aggregated particles are present in solution of laccase *C. hirsutus* 072, and their calculated percent ratio is 85 : 15, respectively, and it does not change in the course of phase transition.

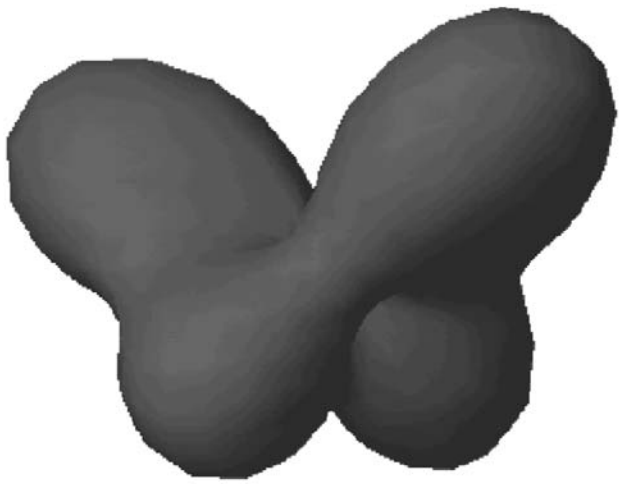

**Fig. 3.** Shape of aggregated particles of laccase 130 Å in diameter calculated using the SASHA modeling package.

The shape of laccase particles attaining 130 Å in diameter was calculated using the SASHA modeling software [29] developed at the Laboratory of small-angle X-ray scattering (Shubnikov Institute of Crystallography, Russian Academy of Sciences). This shape is presented in Fig. 3. Since calculations were based on scattering data of solution in which both monomers and aggregated molecules were present, the shape thus obtained corresponds with some "averaged" particle and is an approximate evaluation of a possible shape of aggregates.

There are three main stages in the process catalyzed by laccases:

– binding of reducing substrate in the T1 pocket with subsequent reduction of T1-$Cu^{2+}$ to $Cu^{+}$;

– intramolecular electron transfer from T1 site to T2/T3 cluster;

– binding and subsequent reduction of a dioxygen molecule to water in the T2/T3 cluster.

The catalytic cycle of laccase is very complicated and assumes mutual transfers of the intermediate enzyme forms accompanied by change in its catalytic activity [1, 30].

Spectroscopic and crystallographic data on copper-containing oxidases suggest a scheme of molecular dioxygen reduction to water via a four-electron mechanism [31]. According to this hypothetical scheme, completely reduced copper ions interact with the dioxygen molecule yielding "peroxide intermediates", which then form a "native intermediate" with all copper ions in the oxidized form. This "native intermediate" can be further reduced and, in contrast, can transfer into the "resting" enzyme form as a result of protonation [32]. A possible mechanism of this transfer obviously accompanied by change in the catalytic activity of laccase is presented in Fig. 4, but there is a lack of exact data in the literature. Studies of phase transitions of laccase allow evaluation of changes in the intermediate enzyme forms, including transfers related with protonation of copper ions; this was one of the goals of the present work.

To evaluate the dynamics of enzyme activity in the course of phase transitions, we studied the temperature effect of laccase activity from 4 to 20°C. Changes in activity of the native and frozen laccase preparations were evaluated during 4 h after cooling to 4°C and thawing, respectively. It should be mentioned that enzyme concentration in the cell was $2 \cdot 10^{-8}$ M in all activity measure-

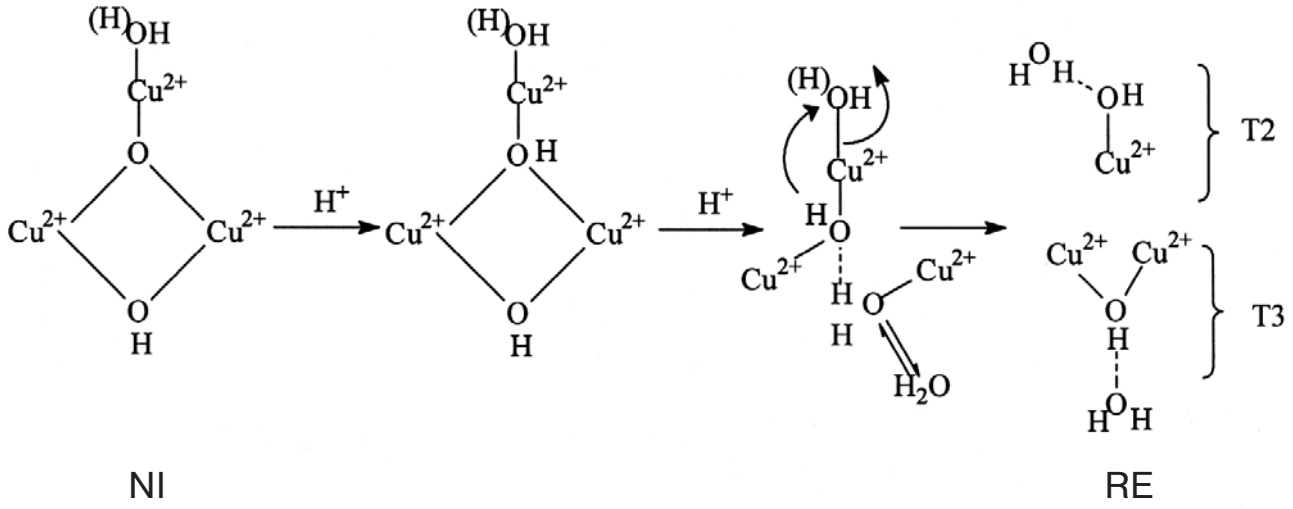


**Fig. 4.** Possible transfer mechanism of laccase forms in solution: NI, native intermediate; RE, resting form of the enzyme [32].

ments. As shown in Fig. 5 (curve *1*), activity of the native laccase preparation cooled to 4°C did not change during subsequent incubation for 4 h at room temperature and was ~50 μmol substrate/min per mg protein, which is in accordance with [33]. However, laccase did not exhibit any enzyme activity immediately after thawing, then during the subsequent 4 h incubation significant activation was observed during the first 40 min, and activity increased up to attaining a plateau after incubation for 160 min, when laccase activity became equal to that of the native preparation (Fig. 5, curve *2*). We suggested that such dynamics of changes in enzyme activity after thawing of laccase preparation is caused either by change in the ratio of monomeric and aggregated enzyme particles in solution or change in the structure of its active site. As shown above, we did not detect change in the ratio of monomeric and aggregated enzyme particles in solution by small-angle X-ray scattering, so the second suggestion seems to be more probable.

To elucidate the effect of phase transition on the active site structure, the state of T1 and T3 centers was studied spectroscopically at 610 and 340 nm, respectively, in the temperature range 12-30°C (Fig. 6). It should be noted that optical absorption of enzyme solution at 610 nm (characteristic wavelength for T1 copper ions as electron acceptors) slightly decreased on increasing temperature (Fig. 6, curve *1*). Changes in this center seem to be either negligible or cannot be detected by this spectroscopic method.

As mentioned above, there are four copper ions surrounded by different ligands and thus having different

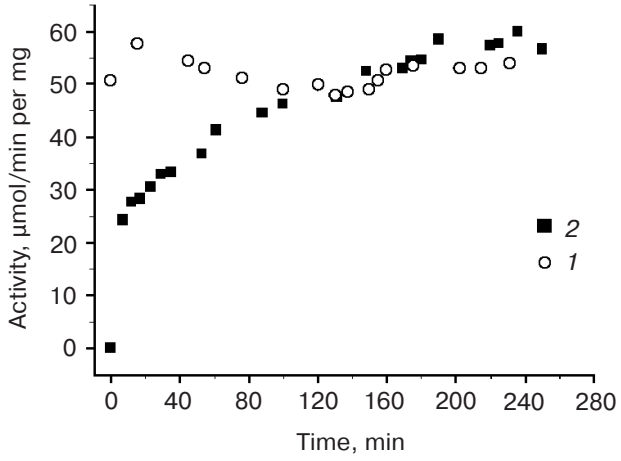


**Fig. 5.** Dynamics of enzyme activity of laccase preparations (0.5 mg/ml): *1*) native laccase after cooling to 4°C and subsequent incubation at room temperature; *2*) laccase after thawing and incubation at room temperature.

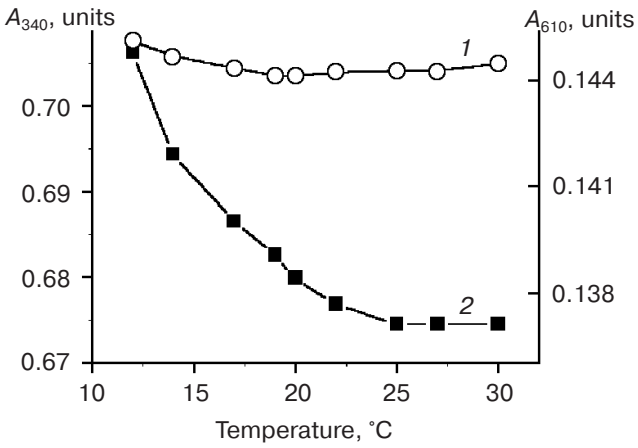


**Fig. 6.** Effect of temperature on spectral characteristics of copper centers of laccase. Changes in optical absorption: *1*) at 610 nm; *2*) at 340 nm.

structure of copper complexes in the active site of laccase. However, the amino acid-composed coordination sphere of copper centers is by itself highly conservative and differs only in the case of T1 copper ion, thus determining the redox potential typical of a certain enzyme. According to our earlier and other data [12, 20], laccase solution is decolorized in the course of freezing. Blue color of enzyme solution is restored after thawing in air; this obviously indicates that the T1 center is changed. The absence of changes in optical absorption spectra at 610 nm beginning from the initial temperature 12°C is probably related with rapid restoration of T1 center to the normal state on temperature increase. Analogous changes were detected for plant laccase [34], but the restoration rate was significantly slower, and this is related with a low redox potential of the T1 center. The main changes possibly occur in the T1 center, and they might be evaluated by EPR spectroscopy.

A significant decrease in optical absorption of the solution was observed at 340 nm (Fig. 6, curve *2*), that is, at the wavelength characteristic for the antiferromagnetic T3 pair. However, the T3 center is EPR silent, and this makes investigation difficult because it is EPR spectroscopy, which provides the main information about rearrangements in the active site of laccases.

Further studies by EPR spectroscopy allowed evaluation of changes in T1 and T2 centers, because EPR spectra of the active site of laccase are actually the sum of spectra of copper centers of the first and second types.

EPR spectra of the native laccase sample and that after thawing were first analyzed. Spectra were recorded at 290 K. However, processing of such spectra was difficult due to broadening of the spectral lines (data not given here). This broadening caused by the averaged anisotropy parameters, *g* and *A* tensors, results from the increased rotational mobility of a complex. When EPR spectra are recorded at room temperature, the hyperfine structure (HFS) is resolved only for T1 copper centers, so HFS of T2 copper centers is not resolved under such conditions.

When EPR spectra are recorded at 77 K, that is, when movement is retarded, HFS clearly manifests itself for T1 as well as for T2 copper centers. EPR spectra of the native laccase (spectrum *3*), immediately (spectrum *1*), and 90 min after thawing (spectrum *2*) recorded at 77 K are presented at Fig. 7. As it can be seen, the EPR spectrum of laccase immediately after thawing differs from that of the native laccase. When the enzyme subjected to freezing/thawing is incubated for 90 min at room temperature, its EPR spectrum (Fig. 7, spectrum *2*) appears to be similar to that of the native laccase. In fact, calculation and analysis of parameters of spin Hamiltonian (table) indicate that the structure of the active site of laccase is drastically changed when the frozen enzyme preparation is stored for a long time. Parameters of the EPR spectrum of the native laccase correspond to those described in [1, 12]. Increased $g_{\|}$ and decreased $A_{\|}$ parameters of EPR spectra recorded immediately after thawing of a frozen sample indicate that the spatial structure of T1 and T2 copper centers is significantly changed. However, these changes appear to be reversible, and just after 90 min the active site structure becomes practically the same as that of the native laccase (Fig. 7, spectra *2* and *3*). This is in accord with the data on enzyme activity of the studied laccase preparation. As shown above, laccase does not

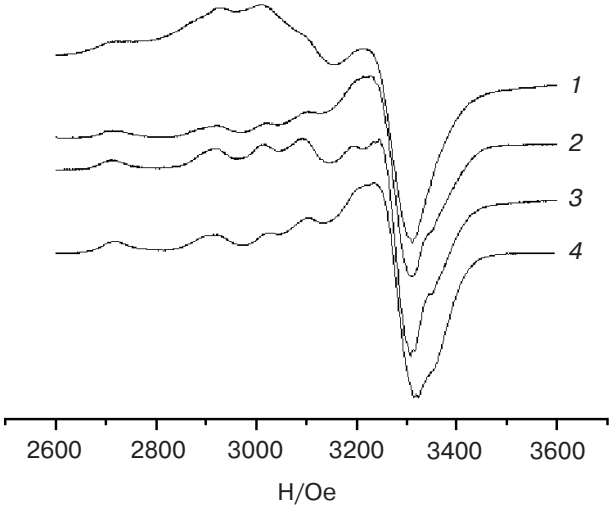


**Fig. 7.** EPR spectra of laccase from *C. hirsutus* at 77 K: *1*) immediately after thawing; *2*) 90 min after thawing; *3*) native enzyme cooled to 277 K; *4*) the same heated to 298 K.

Parameters of spin Hamiltonian of EPR spectra of T1 and T2 copper centers of laccase from *C. hirsutus*

| Preparation | $g_{\|}$ | | $A_{\|}$ ($10^{-3}$ $cm^{-1}$) | | Concentration ratio of T2/T1 copper centers |
|---|---|---|---|---|---|
| | T1 center | T2 center | T1 center | T2 center | |
| Native laccase* | 2.19 | 2.24 | 9.4 | 19.3 | 1 |
| Laccase immediately after thawing | 2.25 | 2.32 | 7.7 | 11.4 | 3 |
| Laccase 90 min after thawing | 2.19 | 2.24 | 9.4 | 19.3 | 0.8 |

* Not subjected to freezing.

exhibit enzyme activity immediately after thawing, but incubation for the first 90 min at room temperature results in almost complete restoration of the enzyme activity (Fig. 5).

Concentration ratio of T1 and T2 copper centers in the EPR spectrum of the native laccase is 1 (table); consequently, the contributions of EPR spectra of T1 and T2 copper centers in the combined spectrum may be considered as equal. However, the number of paramagnetic T2 centers becomes significantly higher than that of T1 centers immediately after thawing of the sample. Similar results were obtained for fungal laccase *T. ochracea* [20]: HFS parameters of T1 copper center are 2.20 ($g_\parallel$) and $9.4 \cdot 10^{-3}$ $cm^{-1}$ ($A_\parallel$) and those of T2 center are 2.24 and $19.4 \cdot 10^{-3}$ $cm^{-1}$, respectively; the concentration ratio of T1/T2 copper centers is 0.8. Such "anomalous" characteristics are rationalized by autoreduction of T1 center of laccase caused by aggregation of laccase molecules in the frozen state; as a result, diamagnetic $Cu^+$ is not detected in EPR spectra [20]. Nonetheless, in this study we have shown that after thawing the ratio of monomeric and aggregated laccase forms remains the same; however, laccase preparation, which does not exhibit any enzyme activity immediately after thawing, restores it during incubation for 180 min at room temperature. "Anomalous" HFS parameters of the EPR spectra return to the values typical of the native laccase preparations during the same time period. A change in the spatial structure of T2/T3 cluster most probably causes change in the ratio of T1 and T2 paramagnetic centers in the laccase molecule in the course of freezing/thawing: significant changes in the T3 center can be seen in optical absorption spectra. It was shown [16, 35] that when sodium azide, which intensively interacts with T2/T3 cluster, is added to laccase, parameters of the spin Hamiltonian for T2 copper center are changed significantly: $g_\parallel$ increases and $A_\parallel$ markedly decreases as in our case.

So, the data indicate that in the course of phase transition and subsequent attaining equilibrium (180 min incubation at room temperature), the structure of all the three copper centers is rearranged. The presence of aggregated enzyme forms in solution is detected before phase transition, the ratio of monomeric and aggregated laccase particles being unchanged after thawing and being the same during subsequent incubation of the enzyme. Nonetheless, the studied preparation does not exhibit enzyme activity immediately after thawing; this may be caused by formation of intermediate enzyme forms: their subsequent transformations change the active site and thus restore its catalytic activity. For better understanding of reversible changes in the structure of copper complexes of laccase detected by us during its freezing/thawing, studies by the new methods and approaches are required.

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