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# Laccases from Basidiomycetes: Physicochemical Characteristics and Substrate Specificity towards Methoxyphenolic Compounds

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**Abstract**—Laccases from the Basidiomycetes *Coriolus hirsutus*, *Coriolus zonatus*, *Cerrena maxima*, and *Coriolisimus fulvocinerea* have been isolated and purified to homogeneity and partially characterized. The kinetics of oxidation of different methoxyphenolic compounds by the fungal laccases has been studied. As laccase substrates, such methoxyphenolic compounds as 4-hydroxy-3,5-dimethoxycinnamic acid (sinapinic acid), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), and 2-methoxyphenol (guaiacol) were used. The stoichiometries of the enzymatic reactions were determined: guaiacol and sinapinic acid are one-electron donors and their oxidation apparently results in the formation of dimers. It was established that $k_{cat}/K_m$, which indicates the effectiveness of catalysis, increases in the series guaiacol, ferulic acid, and sinapinic acid. This fact might be connected with the influence of substituents of the phenolic ring of the substrates. This phenomenon was established for fungal laccases with different physicochemical properties, amino acid composition, and carbohydrate content. This suggests that all fungal laccases possess the same mechanism of interaction between organic substrate electron donors and the copper-containing active site of the enzyme and that this interaction determines the observed values of the kinetic parameters.

*Key words*: laccase, methoxyphenolic compounds, ferulic acid, sinapinic acid, guaiacol, catalytic efficiency

Laccase (monophenol, dihydroxyphenylalanine: oxygen-oxygenase, EC 1.14.18.1) is a copper-containing oxidase that catalyzes the oxidation of various aromatic compounds (phenol derivatives, ascorbic acid, and also some inorganic compounds [1, 2]) to molecular oxygen. The reduction reaction at the active site of the enzyme proceeds without elimination of oxygenic intermediates and leads to water molecule formation, as demonstrated by electrochemical and EPR studies [1, 2]. However, the mechanism of action of the enzyme is not fully clear due to the uncertainty in the nature of the transformation of the electron donating organic substrate as well as to uncertainty of the nature of the rate-limiting stage of the catalytic process. There are two main concepts: according to one, electron transport from organic substrates to the enzyme is not a limiting stage of the catalytic process [3], according to the other the kinetic parameters depend on the oxidation–reduction properties of the organic substrate [4-6]. Investigations of organic substrate transformations are complicated by the fact that in most cases one substrate molecule transforms into several reactive products of oxidation. This complicates the interpretation of kinetic data due to the possible presence of nonenzymatic, oxidation, and polymerization processes [7-11]. Thus, we undertook the comparative study of the kinetic of substrate oxidations that on one hand form a series of compounds with changing properties and, on the other hand, whose reactions of enzymatic oxidation proceed with a minimal number of additional processes (rapid polymerization of products, etc.).

Sinapinic and ferulic acids are methoxyphenolic compounds. The aromatic ring in their molecules conjugates with a double bond. This distinguishes them from the simplest phenolic compounds and phenols containing aliphatic substituents of the aromatic ring. The aim of this work was to determine the influence of phenolic ring substituents (including the methoxy group and double bond-containing substituents) on kinetic parameters of the oxidation reaction by comparing the parameters for sinapinic and ferulic acids and guaiacol (the simplest

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methoxyphenolic compound) as well as other methoxyphenolic compounds. It should be noted that the other reason for this particular choice of substrates was that these substrates can be considered as model lignin components (fungi laccases take part in bioconversion of lignin). Laccases synthesized by different Basidiomycetes were used for generalization of the kinetic data.

## MATERIALS AND METHODS

Laccases were isolated from the culture media of the Basidiomycetes *Coriolus hirsutus*, *Coriolus zonatus*, *Cerrena maxima*, and *Coriolisimus fulvocinerea*. The enzymes were purified by methods reported previously [12-14]. Homogeneity of preparations was monitored by electrophoresis and HPLC [15, 16]. Isoelectric points were determined by isoelectric focusing [15]. Amino acid composition and the content of carbohydrates were determined by methods described in [17] and [18], respectively. Concentrated enzyme solutions were stored at −20°C. For kinetic experiments, the solutions were diluted 50-200-fold with distilled water. Enzyme concentrations were measured by the Lowry method [19], and enzyme stability was studied as described previously [14].

Ferulic and sinapinic acids, pyrocatechol, and serotonin were obtained from Sigma (USA); guaiacol, vanillic alcohol, vanillic acid, eugenol, and 3-(3,4-dihydroxyphenyl)-L-alanine were from Fluka (Switzerland); hydroquinone, vanillin, and homovanillic acid were from Merck (Germany).

Citric acid (Mallinckrodt Chemical, USA) and sodium hydroxide (Reakhim, Russia) were used for preparation of buffer solutions.

All spectrophotometric measurements were performed with a Hitachi 740 spectrophotometer (Japan) at 25°C.

**Determination of molar absorption coefficients.** Absorption spectra of substrates were recorded in 0.02-0.05 M citric buffer; pH was varied in the range 3.5-4.5. The substrate concentration in the spectrophotometer cell was 5-100 μM. The reaction was initiated by adding 10 μl of the enzyme (concentration in the spectrophotometer cell 100-300 ng/ml). Absorption coefficients for oxidation of ferulic and sinapinic acids (difference coefficient between substrate and product absorption) were calculated using the ratio $(A_0 - A_1)/c_0$ and the molar absorption coefficient of the guaiacol product from the ratio $A_1/c_0$, where $A_0$ is the absorption of the solution at time zero (substrate solution in the spectrophotometer cell before the enzyme addition), $A_1$ is the absorption of the solution at the time corresponding to complete conversion of the substrate, and $c_0$ is the initial concentration of the substrate in the cell.

**The reaction stoichiometry.** The measurements were performed by the amperometric method in an electrochemical cell using a Clark-type electrode at room temperature. Into the electrochemical cell with enzyme-containing buffer solution, 10 μl of the substrate stock solution was added so that the resulting substrate concentration was 100 μM. The enzyme concentration in the electrochemical cell was 125 ng/ml (2.27 nM). The initial rate of oxygen decrease was measured. Simultaneously, the initial rate of decomposition of organic substrate (sinapinic acid oxidation) was measured using a spectrophotometer under the same conditions (concentrations of reagents, temperature, and pH). The stoichiometry was calculated as the ratio of the rate of organic substrate conversion to the rate of oxygen decrease.

**Catalytic activity measurements.** Initial velocities of the enzymatic reactions were measured spectrophotometrically at 25°C for ferulic and sinapinic acids and for guaiacol. The concentrations of stock solutions of substrates used were 0.01 M in ethanol for ferulic and sinapinic acids and 0.05 M in citrate buffer (pH 4.3) for guaiacol. A 10-μl sample of the enzyme stock solution and varied volume (5-20 μl) of the substrate were usually added to 2 ml of citric buffer (enzyme concentration in the spectrophotometer cell was 1-3 nM for different enzymes). The rates of guaiacol, ferulic acid, and sinapinic acid oxidation were registered at 465 nm (product accumulation), 312 nm (substrate diminution), and 307 nm (substrate diminution), respectively. For calculation of kinetic parameters, molar extinction coefficient 5200 $M^{-1}\cdot cm^{-1}$ (absorption of guaiacol oxidation product), 11,000 $M^{-1}\cdot cm^{-1}$ (difference between ferulic acid and its oxidation product absorption), and 16,500 $M^{-1}\cdot cm^{-1}$ (difference between sinapinic acid and its product absorption) were used. For other substrates, the kinetic parameters were determined using a Clark electrode by oxygen consumption.

The Michaelis constant for electron donor and the catalytic constant were determined from Lineweaver–Burk plots.

## RESULTS AND DISCUSSION

In this work laccases from four strains, *Coriolus hirsutus*, *Coriolus zonatus*, *Cerrena maxima*, and *Coriolisimus fulvocinerea*, have been chosen for study. The stains belong to different genera of the Basidiomycetes family and they possess different physiological and biochemical characteristics. The cultivation conditions were individual for each strain used. The laccases synthesized by the stains differ in molecular weight, amino acid and carbohydrate composition, isoelectric points, and stabilities (Table 1). It should be mentioned that, due to amino acid composition, the laccase from *Coriolus zonatus* differs very much from another laccases: it contains an unusual amount of glycine (102 residues per protein molecule).

**Table 1.** Some characteristics of laccases

| Laccase source | Molecular weight, kD | Carbohydrate content, % | Carbohydrate composition | p*I* | Inactivation half time at 50°C, h |
|---|---|---|---|---|---|
| *Coriolus hirsutus* | 55 | 12 | N-acetylglucosamine, mannose | 4.0 | 72 |
| *Coriolisimus fulvocinerea* | 54 | 32 | N-acetylglucosamine, mannose, galactose | 3.5 | n.d. |
| *Coriolus zonatus* | 62 | 10 | N-acetylglucosamine, mannose, galactose | 4.6 | 56 |
| *Cerrena maxima* | 57 | 13 | N-acetylglucosamine, mannose, galactose, N-acetylgalactosamine | 3.5 | 52 |

Note: n.d., not determined.

For comparison, *Coriolus hirsutus* and *Coriolisimus fulvocinerea* laccases contain 23 and 34 residues of glycine per molecule, respectively. The carbohydrate compositions of the studied enzymes were usual for fungal laccases. However, 32% of the carbohydrate in the *Coriolisimus fulvocinerea* laccase molecule is not typical of fungi laccases, but the composition is typical of arbocetum laccases. The isoelectric points indicate that the studied enzymes are so-called "acidic laccases".

Two methods of laccase activity measurements are most widely accepted: the amperometric method using a Clark electrode (oxygen consumption measurements) and the spectrophotometric method. Both methods were used in this work.

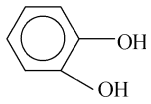
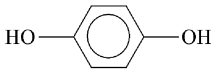
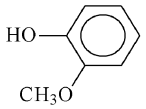
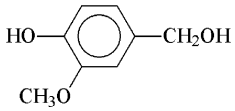
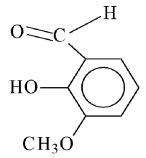
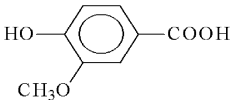
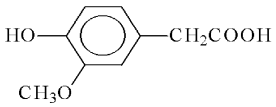
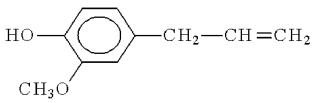
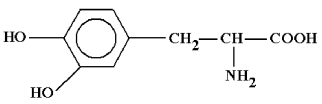
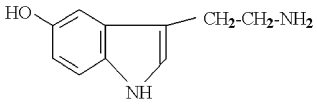
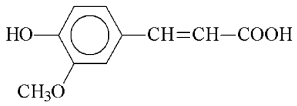
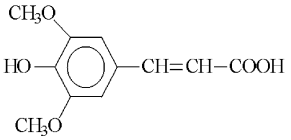
Study of the total kinetic curve is very difficult for many methoxyphenolic substrates (see introductory section). However, the amperometric method of reaction rate determination (Clark electrode) can be used to determine the Michaelis and catalytic constants using initial rates. Comparison of catalytic constant values measured by amperometry for different substrates provides only an estimate since catalytic constants depend on reaction stoichiometry. Thus, different amounts of an organic substrate are used per mole of oxygen in different reactions. In contrast to the catalytic constant, the Michaelis constant does not depend on reaction stoichiometry, so comparison of Michaelis constants for the reactions with different substrates can be relevant. The Michaelis constant values were determined for a large series of phenolic substrates (Table 2). The Michaelis constants for the majority of the tested substrates were 80-200 μM. For *o*-vanillin, the Michaelis constant is much higher. That might be due to the presence of the electronegative aldehyde group in the ortho position of the benzene ring. It was also found that ferulic and sinapinic acids have lower Michaelis constants than the other substrates. Clearly, conjugation of C=C bond with the phenolic ring leads to such a decrease in the effective Michaelis constant value.

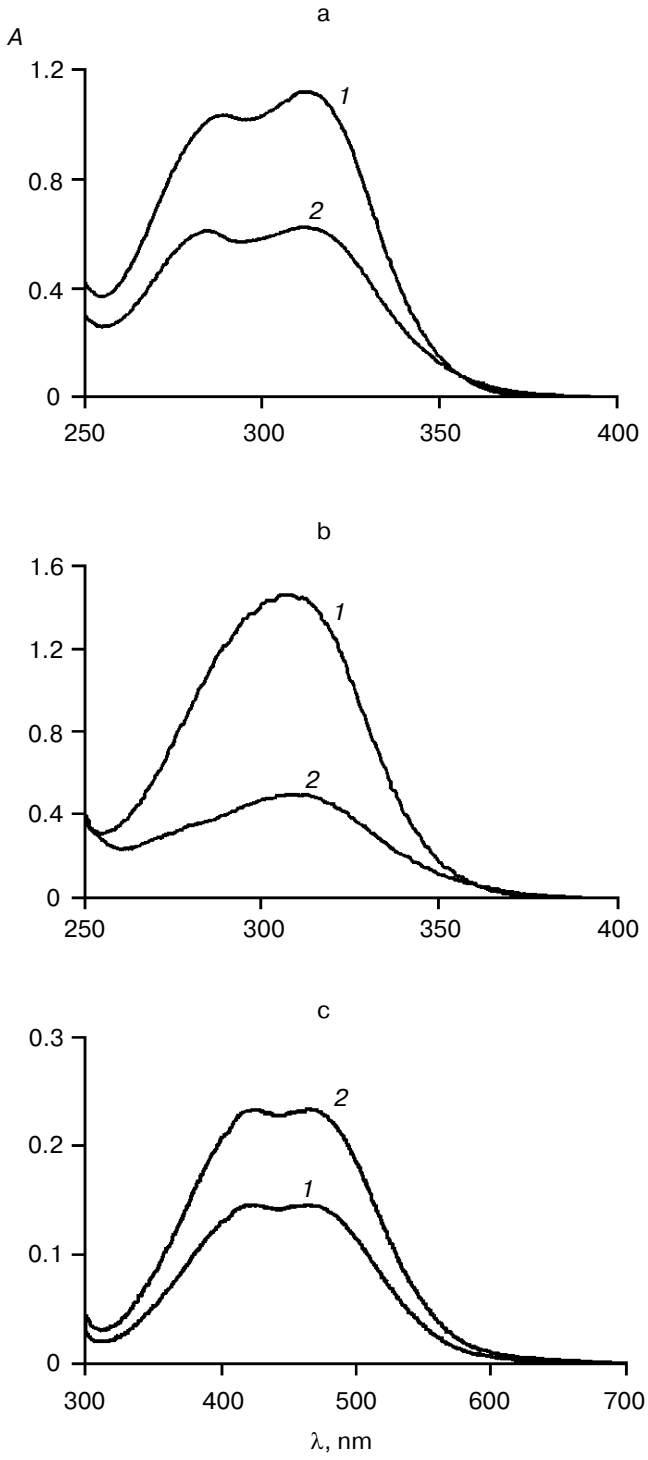
Based on the results of measurements of Michaelis constants, three substrates (sinapinic acid, ferulic acid, and guaiacol) were selected for detailed study of the effectiveness of the catalysis. Analysis of spectra changes during the oxidation reaction showed that the rate of the reaction of ferulic and sinapinic acids can be measured by their decrease, and the rate of guaiacol oxidation can be measured by accumulation of its colored product (see figure). It should be noted that sinapinic acid oxidation proceeds as a multiple stage process that includes enzymatic and non-enzymatic stages. The stages were not analyzed in detail in this work. However, it was found that the consecutive reactions are separated in time, and this allows determination of the catalytic parameters of the first stage. The spectra change during the sinapinic and ferulic acid oxidations using laccase and the type of kinetic curves were shown to be independent of the enzyme source.

The determination of reaction stoichiometry is a very important step in kinetic investigation. In the case of laccase, the combination of two methods, amperometry and spectrophotometry, can be used to determine stoichiometry (see "Materials and Methods"). It has been established that the stoichiometry of the reaction of ferulic and sinapinic acids (and for comparison for guaiacol) corresponds to the ratio of one molecule of oxygen per four molecules of the electron donor. This stoichiometry suggests that ferulic and sinapinic acids are one-electron donors. Consequently, the products of the reaction should be dimeric. The identical stoichiometry for the two substrates allows comparison of the kinetic parameters of the catalytic transformation correctly with no information about the structure of products formed.

Increase of $k_{cat}$ and decrease of $K_m$ were observed in the series guaiacol, ferulic acid, and sinapinic acid. The

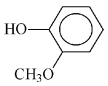
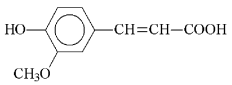
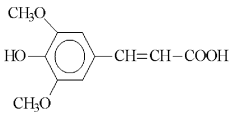
**Table 2.** Michaelis constants for different phenolic substrates for laccase from *C. zonatus* measured by amperometry

| Substrate | Substrate structure | $K_m$, μM |
|---|---|---|
| Pyrocatechol |  | 197 |
| Hydroquinone |  | 86 |
| Guaiacol |  | 91 |
| Vanillic alcohol |  | 85 |
| *o*-Vanillin |  | 3900 |
| Vanillic acid |  | 165 |
| Homovanillic acid |  | 102 |
| Eugenol |  | 80 |
| L-Dioxyphenyl-alanine |  | 180 |
| Serotonin |  | 118 |
| Ferulic acid |  | 25 |
| Sinapinic acid |  | 7 |




Typical changes of UV/visible absorption spectra during oxidation of organic substrates by fungal laccase (experimental conditions: 0.02 M citrate buffer, pH 4.5; concentration of laccase from *C. zonatus* is $1.5 \cdot 10^{-9}$ M): a) decrease in ferulic acid content (spectrum before enzyme addition (*1*); spectrum 5 min after starting the reaction (*2*)); b) decrease in sinapinic acid content (spectrum before enzyme addition (*1*); spectrum 5 min after starting the reaction (*2*)); c) accumulation of guaiacol product (spectrum 5 min (*1*) and 10 min (*2*) after starting the reaction).

**Table 3.** Kinetic parameters of oxidation reactions

| Substrate | *Coriolus hirsutus* | | | *Coriolisimus fulvocinerea* | | | *Coriolus zonatus* | | | *Cerrena maxima* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $K_m$, μM | $k_{cat}$, $sec^{-1}$ | $k_{cat}/K_m$ | $K_m$, μM | $k_{cat}$, $sec^{-1}$ | $k_{cat}/K_m$ | $K_m$, μM | $k_{cat}$, $sec^{-1}$ | $k_{cat}/K_m$ | $K_m$, μM | $k_{cat}$, $sec^{-1}$ | $k_{cat}/K_m$ |
| Guaiacol  | 65 | 420 | 6.5 | 70 | 95 | 1.4 | 90 | 70 | 0.77 | 255 | 210 | 0.93 |
| Ferulic acid  | 31 | 490 | 15.3 | 50 | 110 | 2.2 | 25 | 100 | 4 | 34 | 285 | 8.40 |
| Sinapinic acid  | 17 | 625 | 38 | 21 | 150 | 7.1 | 10 | 160 | 16 | 13 | 360 | 27.7 |

second order constant $k_{cat}/K_m$ indicates the effectiveness of the catalysis. Comparison of $k_{cat}/K_m$ for the considered substrates shows decrease of catalysis effectiveness by 5-20-fold, and the tendency is the same for all laccases studied (Table 3).

It is well known that the laccase oxidation reaction proceeds by a radical mechanism. The mechanism of the laccase oxidation includes electron detachment from the substrate molecule producing a phenoxyl radical [1, 2]. Considering these facts, we suggest that the kinetic phenomenon described should be explained using the conceptions of inductive and mesomeric effects of substituents. Ferulic and sinapinic acids differ from guaiacol by presence of a substituent in the *para* position of the benzene ring. The hydrogen atom has no mesomeric effect, and the CH=CH–COOH group has a negative mesomeric effect. Conjugation of a double bond with the phenolic ring stabilizes the forming radical by delocalization of the unpaired electron. This could be the reason of the increased catalytic effectiveness. The influence of the positive mesomeric effect of an additional methoxy group could explain the increase of catalytic effectiveness for sinapinic and ferulic acids.

The experimental results obtained in this work suggest that the rate constants of the interaction between organic substrate and the enzyme active site contribute to the general equation of the reaction rate and to the effective kinetic parameters observed. Perhaps one of the stages is rate limiting. The experimental data were obtained for fungal laccases from different sources. This suggests that the regularities of catalysis described are general for fungal laccases. The data of this work can be considered as preliminary for further study of laccase substrate specificity (including identification of products of enzymatic oxidation). Moreover, the study of the effect of enzyme activators and inhibitors might clarify the features of the enzymatic mechanism.

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