# ENZYMATIC OXIDATION OF LIGNIN AND COMPOUNDS MODELING IT.

# VI. ANALYSIS OF THE PRODUCTS OF LACCASE OXIDATION OF LIGNINS

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It has been shown that the processes involved in the formation of quinones and the recombination of peroxide radicals take place with the same rate constants. The results obtained give grounds for assuming that the formation of quinones is the result of the recombination and disproportionation of peroxide radicals. A sequence of the reactions taking place in the enzymatic oxidation of lignin is suggested.

Recently, a number of publications devoted to the oxidative enzymolysis of lignin using the enzyme laccase have appeared. It has been shown [1] that laccase III depolymerizes the water-soluble fraction of lignin, while laccases I and II are capable of polymerizing the products of the oxidation of lignin. It has been established [2] that the action of laccase on ground wood lignin (GWL) lead to an increase in the amount of carboxy groups and to a rise in the water solubility of the oxidized lignin. It is assumed that the changes described are connected with radical reactions taking place under the action of laccase in the side chains of the lignin. The action of laccase on GWL leads to an increase in the number of phenolic hydroxy groups, which may be due to the cleavage of aryl alkyl ether bonds [3].

In the present paper we give the results of physicochemical analyses of the products of the laccase oxidation of lignin.

In the laccase oxidation of lignin a substantial change is observed in the absorption of the substrate both in the UV and in the visible regions of the spectrum. The action of laccase on lignin leads to the appearance of a band in the 315-nm region and a shoulder in the 325- to 330-nm region in the UV absorption spectrum of lignin.

The enzymatic oxidation of lignin is accompanied by an intensive red coloration of the solution. Measurement of the absorption of the lignin oxidation products relative to the solvent (direct spectrum) is difficult because of the high rate of the process and the small changes in the density of the solution, which is apparently connected with the low extinction coefficient of the products formed. To avoid the above-mentioned difficulties, we used the method of difference spectrophotometry in which the absorption spectra of the lignin oxidation products were recorded relative to the initial substrate under transmission conditions, which permits an increase in the sensitivity of the method and enables kinetic measurements to be performed.

In the difference transmission spectrum of the products of the enzymatic oxidation of lignin there are bands in the 28,000-27,000 $cm^{-1}$ (360-370 nm) and 24,000-19,000 $cm^{-1}$ (420-560 nm) regions (Fig. 1). Absorption in the 350- to 370-nm region is usually connected with the formation of the α-carbonyl groups in the lignin molecule. However, quinone methides and phenylcoumaran structures usually absorb in this region [4] and, therefore, the assignment of the 360- to 370-nm band is not unambiguous.

An absorption band appears in the 510-nm region as the result of the aerobic oxidation of lignin in the presence both of laccase and of peroxidase, while the addition of hydrogen peroxide inhibits the formation of this maximum. In the opinion of Steelink [5], the red band of lignin oxidation products is given by quinoid compounds (of the type of p-quinones) formed as the result of the disproportionation of phenoxyl radicals.

The effective rate constant of the formation of chromophores absorbing in the 510-nm region is $1.3 \cdot 10^{-3}$ $sec^{-1}$ both in the case of oxidation in the presence of laccase and in

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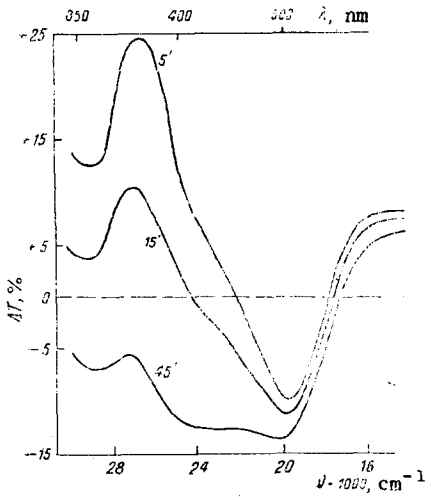


Fig. 1

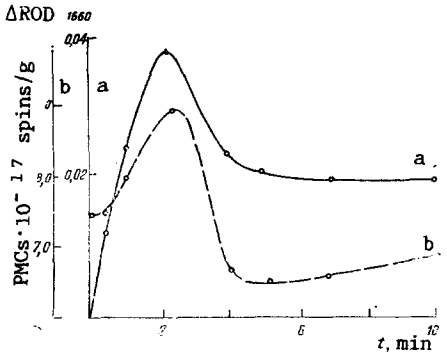


Fig. 2

Fig. 1. Difference transmission spectrum of the products of the laccase oxidation of lignin. The numbers attached to the curves are the reaction times.

Fig. 2. Kinetic curves of the change in the relative optical density at $\nu = 1660\ cm^{-1}$ (a) and of the concentration of PMCs (b) during the laccase oxidation of lignin.

the presence of peroxidase without $H_2O_2$. The constant obtained practically coincides with the effective rate constant of the quenching of chemiluminescence ($1.4 \cdot 10^{-3}\ sec^{-1}$), recorded under identical conditions of the oxidation of lignin [6]. The agreement of the values of the rate constants gives grounds for assuming that the quinoid structure (p-quinones according to [5]) formed in the enzymatic oxidation of lignin are the products of the recombination and disproportionation of peroxide radicals and not phenoxyl radicals, as stated in [5].

In order to investigate the kinetics of the initial stage of the enzymatic oxidation of lignin, we used the method of rapidly freezing samples at the temperature of liquid nitrogen and sublimation under reduced pressure. This method permits the reaction to be stopped at any moment of time and enables dry preparations of oxidized lignin to be obtained for further complex investigation by physicochemical methods of analysis.

The results obtained by IR spectroscopy showed that in the initial stages of the oxidative enzymolysis of lignin ($t < 2.5$ min) the formation takes place of carbonyl groups of the quinone type, as was shown by a rise in the relative optical density (ROD) in the 1660-1680 $cm^{-1}$ region. Further reaction led to a fall in the concentration of quinoid structures (Fig. 2a) to a steady level. The prolonged oxidation of lignin ($t > 30$ min) was accompanied by an accumulation of carboxylic structures, judging from the rise in density in the 1720 $cm^{-1}$ region.

The initial stage of the enzymatic oxidation of lignin was characterized by a sharp increase in the concentration of paramagnetic centers (PMCs) and then by a fall in the concentration of PMCs to a practically steady state (Fig. 2b). The maximum concentrations of quinones and of paramagnetic centers coincide with respect to reaction time.

Summarizing the results of the investigations performed, it may be assumed that the process of oxidative enzymolysis of lignin takes place by the following scheme: initial lignin → phenoxyl radical → peroxide radical → excited quinone → stable radical.

The formation of the phenoxyl radical in the enzymatic oxidation of lignin has been shown previously [5]. The interaction of the phenoxyl radical with oxygen (formation of a peroxide radical) and the formation of excited intermediates has been shown in [6]. A stable spatially hindered phenoxyl radical may have the structure suggested in [8].

## EXPERIMENTAL

The spectra in the UV and visible regions of the products of the oxidation of lignin and also the kinetic characteristics of the reaction were recorded on a Specord UV-VIS spec-

trophotometer. For recording the difference spectra, a solution of lignin without the enzyme was used as the comparison solution. The effective rate constants of the oxidation of lignin were calculated according to [9].

Dry samples of oxidized lignin were molded with KBr (4:500), and IR spectra were taken on a Specord 75 IR double-beam spectrophotometer in the frequency range of 4000-500 $cm^{-1}$. To calculate relative optical densities (RODs), the 1140 $cm^{-1}$ band was used as internal standard. In Fig. 2, the difference in the $ROD_{1660}$ between the oxidized and initial lignin preparations has been plotted along the axis of ordinates.

The changes in the concentration of paramagnetic centers were recorded on a EPA-2M ESR spectrometer.

Dioxane lignin was obtained from pinewood by the method described in [7]. The oxidation of the lignin was carried out under thermostated conditions at a temperature of 25.0 ± 0.5°C. The solvent used was a mixture of dioxane and water in a ratio of 5:1 by volume. As oxidation catalysts were used pure commercial preparations of peroxidase (Reanal RZ 0.6; activity by the o-dianisidine method 350-500 units/mg, Hungary), and type A laccase ($A_{608}/A_{280}$ = 18), both provided by the Institute of Biochemistry of the Academy of Sciences of the Armenian SSR. The start of the reaction was the addition of the enzyme to the system, the time from mixing to the beginning of recording being 30 sec.

To obtain dry preparations of oxidized lignin we have used for the first time the method of rapidly stopping the reaction by freezing the solution in liquid nitrogen ($t° = -196°C$). The enzymatic oxidation of lignin was performed in quartz cells which, after the lapse of a predetermined interval of time from the beginning of the reaction, were placed in a Dewar vessel with liquid nitrogen. After the complete freezing of the solution, the samples were subjected to lyophilization in vacuum until the solvent had been evaporated off completely. Before the performance of analyses, the samples were placed in a vacuum-drying chest and were dried additionally at 105°C for an hour.

Control experiments showed insignificant differences of the initial and frozen samples. IR spectroscopy showed some broadening of the absorption band in the 3400-3200 $cm^{-1}$ region, which is connected with the formation of additional hydrogen bonds in the lignin macromolecules.

## EXPERIMENTAL

1. It has been shown that the processes involved in the formation of quinones and the recombination of peroxide radicals take place with identical rate constants. On the basis of the results obtained, it may be assumed that the formation of quinones is the result of the recombination and disproportionation of peroxide radicals.

2. A sequence of reactions taking place in the enzymatic oxidation of lignin is suggested.

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