ENZYMATIC OXIDATION OF LIGNIN AND COMPOUNDS MODELING IT. I. INVESTIGATION OF THE PEROXIDASE OXIDATION OF LIGNIN BY THE CHEMILUMINESCENCE METHOD

V. A. Strel'skii and É. I. Chupka

UDC 577.158.5:535.379

In the enzymatic oxidation of lignin, superweak fluorescence is detected which confirms the radical mechanism of peroxidase-catalyzed oxidation. The rate constants of the recombination and disproportionation of the radicals have been calculated. Both the degradation and the partial polymerization of the initial substance have been established by the gel filtration of the lignin oxidation products.

Two enzymes — laccase and peroxidase [1], belonging to the constitutional enzymes — are responsible for the natural oxidative degradation of lignin. The enzymatic oxidation of lignin and compounds modeling it by peroxidase has been studied by the methods of ESR and UV spectroscopy and it has been shown that the lignin macromolecules undergo enzymatic degradation by a mechanism analogous to that for model compounds [2]. The kinetics of the enzymatic oxidation of lignin, and also the radical mechanism of this process had not actually been studied, although they are of great theoretical and practical value. We have now studied the kinetics of the peroxidase oxidation of lignin, and also the influence of oxidation on the change in the molecular mass of the substance under investigation, by the chemiluminescence method.

The oxidation of ethanol lignin by the peroxidase—H_2O_2 system gives rise to luminescence, confirming the opinion of the free-radical nature of the enzymatic oxidation of lignin. When the substrate is added to the system the intensity of the chemiluminescence rises sharply, reaching a certain maximum, after which the intensity of the emission falls exponentially. The decay of the chemiluminescence is satisfactorily described by the equation

$$I_t = I_0 e^{-k^* t},$$

where I_t is the intensity of the chemiluminescence at time t, I_0 is the initial intensity of the chemiluminescence, and k* is the effective rate constant of the decay process.

A typical kinetic curve of the luminescence during the enzymatic oxidation of lignin is shown in Fig. 1.

As a quantitative characteristic of the luminescence we used the intensity I and the total amount of light ΣS, which is numerically equal to the area included under the chemiluminescence decay curve. In this case, the total amount of light ΣS can be used as an index of the depth of oxidation of the substrate. It is obvious that the greater the total amount of light emitted the larger is the number of emitting reaction centers that arises during the oxidation of the lignin. Figure 2 shows the change in the intensity and in the total amount of light of the luminescence as functions of the concentration of the substrate (the concentrations of the enzyme and of hydrogen peroxide were constant and equal to 10^{-5} and $9 \cdot 10^{-2}$ M, respectively). As follows from the results given, these magnitudes change synchronously. These results indicate that the number of emitting centers arising in the recombination of radicals grows in proportion to the amount of lignin. In the range of lignin concentrations studied no maxima characterizing a concentration inhibition of the reaction or an inhibition of the accumulation of oxidation products were recorded. A calculation of the effective rate constant of the decay of the chemiluminescence by the formula given above showed that a linear relationship exists between this magnitude and the concentration of

Siberian Scientific-Research Institute of Pulp and Board, Bratsk. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 762—764, November-December, 1982. Original article submitted January 23, 1982.

 0009-3130/82/1806-0726$07.50

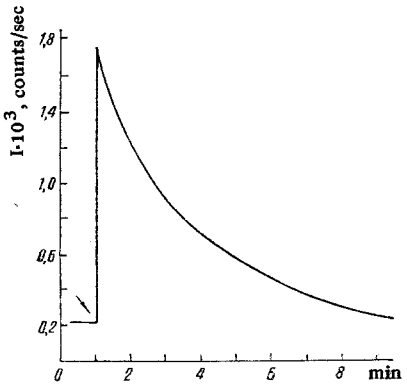

Fig. 1

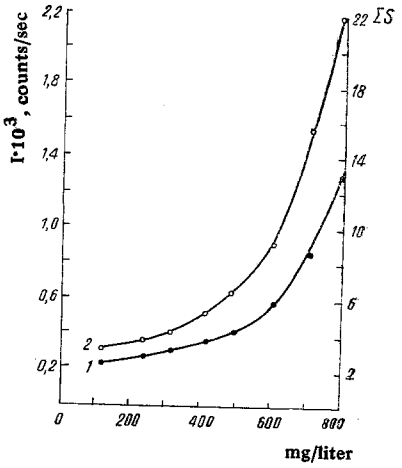

Fig. 2

Fig. 1. Kinetic curve of the chemiluminescence of the enzymatic oxidation of lignin (c_{lig} = 470 mg/liter; $c_{H_2O_2}$ = 0.255 M, C_{enz} = 10^5 M). The arrow shows the moment of addition of the substrate.

Fig. 2. Dependence of the change in the intensity (1) and of the total light (2) of the chemiluminescence in the peroxidase oxidation of lignin on the concentration of the substrate ($c_{H_2O_2}$ = 0.09 M, C_{enz} = 10^{-5} M).

lignin, with $k_1 = 0.9 \cdot 10^{-4}$ $sec^{-1} \cdot liter \cdot mg^{-1}$. The coefficient k_1 can characterize the rate of the processes of recombination and disproportionation of the peroxide and hydroperoxide radicals.

The change in the intensity and of the total amount of chemiluminescence light as a function of the concentration of hydrogen peroxide has a maximum at $2.25 \cdot 10^{-1}$ M. With a further rise in the concentration of hydrogen peroxide the intensity of the luminescence falls, but the total amount of light continues to rise. This phenomenon may be connected with the accumulation of free oxygen, which is liberated in the oxidation process and in high concentrations is an active inhibitor of radical processes [3].

A linear relationship also exists between the effective rate constant k_2^* calculated from the equation given above and the concentration of hydrogen peroxide, with $k_2 = 0.107$ $sec^{-1} \cdot M^{-1}$.

With a variation in the concentration of peroxidase, the curve of the total amount of light has a characteristic maximum at an enzyme concentration of 10^{-6} M, and the dependence of the change in the intensity has a linear nature. Such a relationship indicates a short-lived nature of the lignin-oxidizing process in which, with an increase in the concentration of enzyme, a very rapid formation of peroxide radicals and their subsequent recombination with the formation of inactive oxidation products takes place. With a decrease in the concentration of peroxidase, the rate of recombination of the radicals falls, which is expressed in a reduction of the angle of slope of the initial stage of the kinetic curve of luminescence and an increase in the time of decay of the radiation. The shape of the curve in this case approximates to the curve of luminescence during the oxidation of lignin by hydrogen peroxide in the absence of the enzyme.

The results of the gel filtration of the lignin oxidation products (Fig. 3) showed that in addition to a degradation process accompanied by a fall in the molecular mass of the substrate, its partial aggregation also took place. It was found from the gel filtration curves that in the enzymatic oxidation of lignin 52.7% of the lignin was destroyed, and the yield of products with a higher molecular mass amounted to 13.5%. The partial aggregation of the lignin oxidation products and the appearance of the luminescence under the action of the peroxidase—H_2O_2 makes it possible to suggest that the oxidation of the lignin takes place by a mechanism analogous to the polymerization of such compounds as isoeugenol and α-guaiacylpropane, which are also accompanied by luminescence. It is also possible that, on the interaction of lignin with the peroxidase—H_2O_2 system, structures having a double bond in the β

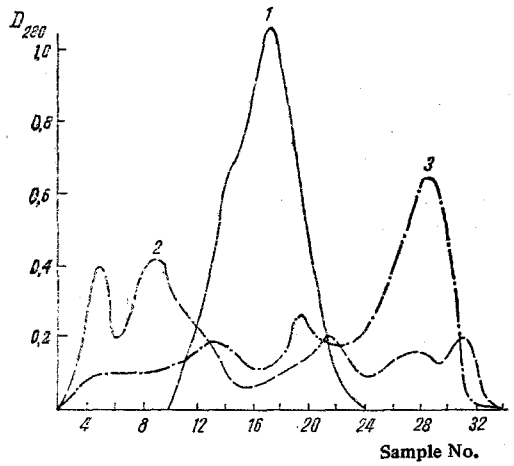

Fig. 3. Gel chromatography of the lignin oxidation products: 1) initial lignin; 2) lignin + H_2O_2; 3) lignin + peroxidase + H_2O_2.

position of the side chain, i.e., structures of the isoeugenol type, which, just like lignin, are characterized by the absence of an induction period of chemiluminescence [4], are oxidized first.

EXPERIMENTAL

A solution of lignin in aqueous ethanol with a volume ratio of 1:1 was subjected to enzymatic oxidation. The ethanol lignin was isolated from the sapwood of a pine tree 100 years old by a method described by Nikitin [5]. A preparation of horseradish peroxidase (Reanal RZ 0.6; activity by the o-dianisidine method 350–500 units/mg) was dissolved in 0.1 M phosphate buffer, pH 5.8. The lignin was oxidized in the presence of hydrogen peroxide at 25.0 ± 0.5°C in a thermostated cell placed in front of a cooled FÉU-84-2 photoelectron multiplier. The signal from the photoelectron multiplier, after amplification in a broad-band amplifier, was fed to a N 306 recording potentiometer. The photorecording apparatus worked under the conditions of a quantum counter. The oxidation products were dissolved in dimethyl sulfoxide and were eluted with the given solvent from a column containing Sephadex G-100. Eluates with a volume of 2.5 ml were photometered on a SF-16 spectrophotometer at λ 280 nm. The degree of conversion was calculated in the following way:

A gel filtration curve was plotted in the coordinates D versus f (sample No.);

the area under the elution curve was taken as 100%; and

the zone of outflow of the initial lignin was excluded from the total area.

The difference in areas between the elution zones of the initial lignin + monomeric fractions and the whole area gives the amounts of condensed oxidation products as a percentage. The calculation for the degraded part of the lignin was carried out similarly.

SUMMARY

In the peroxidase oxidation of lignin, chemiluminescence is observed, which confirms the radical mechanism of this process. The coefficient characterizing the rate of recombination and disproportionation of the peroxide and hydroperoxide radicals is $0.9 \cdot 10^{-4}$ liter·$sec^{-1} \cdot mg^{-1}$. The results of gel filtration have shown that during the enzymatic oxidation of lignin both degradation and the further aggregation of the substrate take place, showing the many-sided nature of this process.

LITERATURE CITED

1. H. Zyr, Planta, 48, 239 (1956).
2. M. Young and C. Steelink, Phytochemistry, 12, 2851 (1973).
3. R. F. Vasil'ev and A. A. Vychutinskii, Dokl. Akad. Nauk SSSR, 142, 3 (1962).
4. R. Nilsson, Acta Chem. Scand., 18, 389 (1964).
5. V. M. Nikitin, The Chemistry of Wood and Cellulose [in Russian], Moscow–Leningrad (1960), p. 103.