# ENZYMATIC OXIDATION OF LIGNIN AND COMPOUNDS MODELING IT. II. A STUDY OF THE PEROXIDASE OXIDATION OF MODEL COMPOUNDS OF THE STRUCTURAL UNIT OF LIGNIN BY THE CHEMILUMINESCENCE METHOD

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An investigation of the radical mechanism of the peroxidase oxidation of monomeric phenols (vanillin, vanillyl alcohol, isoeugenol, and α-guaiacylpropanone) has been made by the chemiluminescence method. It has been established that chemiluminescence is observed in those compounds that contain hydroxy or carbonyl groups on a carbon atom in the α position or have a C=C bond in the β position conjugated with the aromatic ring. The hypothesis has been put forward that peroxide compounds are inhibitors of autocatalytic processes in the oxidation of lignin in the presence of peroxidase.

In the preceding paper [1] we have shown that the peroxidase oxidation of lignin is accompanied by chemiluminescence (CL), which confirms the radical mechanism of the enzymatic oxidation of lignin. The heterogeneous structure of lignin does not permit an unambiguous conclusion to be drawn concerning the reasons for the appearance of and the mechanism of the superweak luminescence. In order to investigate the capacity of the oxidation products for forming excited states, we have studied the enzymatic oxidation of a number of compounds modeling the structural unit of lignin.

In the peroxidase oxidation of a number of monomeric phenols, superweak fluorescence was detected from ethanolic solution of vanillin, vanillyl alcohol, α-guaiacylpropanone, and isoeugenol. As is well known [2], the intensity of the chemiluminescence of the peroxidase reaction rises sharply at the initial moment of the reaction, reaching some maximum level, after which it falls exponentially. T. G. Mamedov et al. [3] have shown that time to reach the maximum of the luminescence and the height of this maximum do not only depend on the concentration of the substrate but also change appreciably at the same concentrations of different substrates. As in the preceding investigation, to characterize the chemiluminescence quantitatively we have investigated the dependence of the change in intensity I and in the total amount of light ΣS on the concentrations of the reactants. In all cases, the background was the luminescence of the peroxidase–$H_2O_2$ complex.

Figure 1 shows the dependence of the change in the intensity and in the total chemiluminescence in the peroxidase oxidation of α-guaiacylpropanone at different concentrations of hydrogen peroxide (the concentrations of enzyme and substrate were constant and equal to $10^{-5}$ and $10^{-2}$ M, respectively). The maxima of the intensity and of the total light emission correspond to a concentration of hydrogen peroxide of 0.09 M. With a further rise in the concentration of oxidizing agent the intensity and the total amount of light emitted fell, which can be explained by an inhibition of the peroxidase reaction by an excess of hydrogen peroxide. The complete synchronicity of the change in these relationships must be mentioned. The same synchronicity is retained when the concentrations of substrate and enzyme are varied. These relationships have no maxima in the range of concentrations studied, which also indicates an inhibition of the reaction by the oxidizing agent. If with a change in the concentration of enzyme the curve has a sigmoid form with passage on to a constant level of intensity and total light, then with a change in the concentration of substrate one must observe a fairly wide interval ($0.8 \cdot 10^{-3}$–$0.5 \cdot 10^{-2}$ M) within which these values scarcely change.

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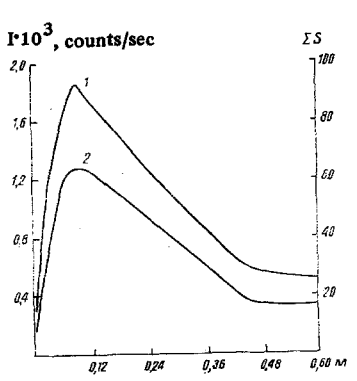



Fig. 1

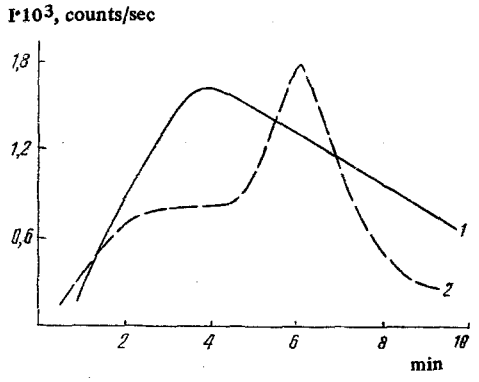


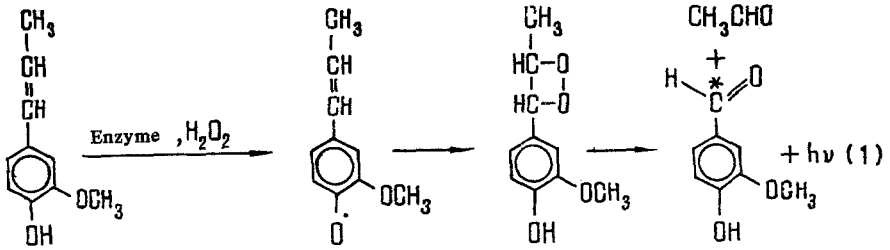

Fig. 2

Fig. 1. Dependence of the change in the intensity (1) and the total amount of chemiluminescence light (2) in the peroxidase oxidation of α-guaiacylpropanone on the concentration of hydrogen peroxide ($c_{enz} = 10^{-5}$ M, $c_{sub} = 10^{-2}$ M).

Fig. 2. Change in the intensity of luminescence during the peroxidase oxidation of vanillyl alcohol at concentrations of hydrogen peroxide less than $6 \cdot 10^{-3}$ M ($c_{enz} = 10^{-5}$ M, $c_{sub} = 10^{-2}$ M): 1) $c_{H_2O_2} > 6 \cdot 10^{-3}$ M; 2) $c_{H_2O_2} < 6 \cdot 10^{-3}$ M.

The oxidation of vanillyl alcohol and of vanillin by the peroxidase—$H_2O_2$ system is similar to the oxidation of α-guaiacylpropanone. Complete synchronicity of the change in the intensity and the total amount of the chemiluminescence light is observed. The maxima of these magnitudes correspond to a concentration of hydrogen peroxide of 0.045 M. At concentrations of the oxidizing agent exceeding $6 \cdot 10^{-3}$ M, an anomaly is observed in the intensity of the luminescence of vanillyl alcohol which was not observed for the other models studied (Fig. 2).

The oxidation of isoeugenol accompanied by radiation takes place both with oxygen in the presence of peroxidase and with hydrogen peroxide in the absence of the enzyme. The natures of the changes in the intensity of the chemiluminescence in the aerobic oxidation of isoeugenol in the presence of peroxidase and in oxidation by the peroxidase—$H_2O_2$ complex are identical. The oxidation of isoeugenol by hydrogen peroxide is accompanied by a more gentle rise and a slow fall of the intensity of luminescence. The intensity and the total amount of light of the luminescence in the oxidation of isoeugenol are considerably higher than for similar concentrations of α-guaiacylpropanone, vanillin, and vanillyl alcohol. With a variation in the concentrations of hydrogen peroxide and of substrate the kinetic curves are not so synchronous as for the substrates mentioned above. The existence of chemiluminescence in the oxidation of isoeugenol makes it possible to assume that the enzymatic oxidation reaction of this substrate takes place through a stage of the formation and decomposition of dioxetanes, as a result of which carbonyl groups in an excited state are formed [4].




Triplet states of carbonyl groups have a tendency to undergo polymerization as a consequence of their dehydrogenating capacity [5], which explains the possibility of the formation of a dehydropolymer in the peroxidase oxidation of isoeugenol.

The oxidation of the other substrates containing an oxygen atom in the α position with respect to the aromatic nucleus is connected with the formation of peroxide radicals ROO·, the subsequent recombination of which leads to the formation of excited ketone molecules [6]. The possibility is not excluded, either, of the participation in the chemiluminescence reaction of the singlet oxygen arising during the peroxidase oxidation of certain substrates [7]. The presence of fairly long induction periods in the peroxidase oxidation of α-guaiacyl propanone, vanillin, and vanillyl alcohol makes it possible to assume the existence of a degenerate branched-chain process. Since the peroxide is the branching factor in the chain reaction, a burst of superweak luminescence may be considered as an indication of the development of a chain process [8]. It is possible to explain from this point of view the anomaly in the intensity of the luminescence on the enzymatic oxidation of vanillyl alcohol.

In the enzymatic oxidation of such monomers as eugenol, ferulic acid, and guaiacol, the luminescence does not exceed that of the background of the peroxidase—$H_2O_2$ system. The addition of guaiacol to the reaction medium causes a suppression of the luminescence of this system. The absence of luminescence in the oxidation of guaiacol shows that chemiluminescence arises only when a monomeric phenol undergoing oxidation contains a side chain. Generalizing what has been said above, it may be concluded that those compounds modeling the structural unit of lignin may take part in the oxidative peroxidase reaction accompanied by chemiluminescence which:

have a hydroxy or a carbonyl group on the carbon atom in the α position with respect to the aromatic ring; and

possess a C=C bond in the β position conjugated with the aromatic ring.

The appearance of chemiluminescence in the oxidation of such compounds as α-guaiacylpropanone, vanillin, vanillyl alcohol, and isoeugenol makes it possible to assume that these compounds are inhibitors of the autocatalytic process in the enzymatic oxidation of lignin.

## EXPERIMENTAL

As models of the structural unit of lignin we used vanillin and vanillic acid of KhCh ["chemically pure"] grade, freshly purified commercial preparations of guaiacol, eugenol, and isoeugenol, and also preparations of α-guaiacylpropanone and ferulic acid obtained as described by Zakis [9]. These compounds were dissolved in ethanol—water (3:2 by volume). A commercial preparation of horseradish peroxidase (Reanal RZ 0.6; activity by the o-dianisidine method 350—500 units/mg) was prepared in 0.1 M phosphate buffer, pH 5.8. The oxidation of the phenols was carried out in the presence of hydrogen peroxide at 25 ± 0.5°C in a thermostated cell placed in front of a cooled FÉU-84-2 photocathode. The photorecording apparatus worked under quantum-counting conditions. The signal from the photoelectron multiplier, after amplification in a broad-band amplifier, was fed to a N-306 two-coordinate recording potentiometer.

## SUMMARY

It has been established as the result of the investigations performed that chemiluminescence is observed in the peroxidase oxidation of such monomeric compounds modeling the structural unit of lignin as have a hydroxy or carbonyl group on a carbon atom in the α position to the aromatic ring or a conjugated C=C bond in the β position and conjugated with it. The enzymatic oxidation of isoeugenol most probably takes place through a stage of the formation and decomposition of dioxetanes, as a result of which energy sufficient to convert the carbonyl group into the excited state is liberated.

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# SYNTHESIS OF BIOLOGICALLY ACTIVE ANALOGS OF LULIBERIN WITH SHORTENED AMINO ACID SEQUENCES

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Two new analogs of the releasing factor of the luteinizing hormone with shortened amino acid sequences have been synthesized by the methods of classical peptide chemistry. The influence of the preparations on the action of chorionic gonadotropin and on the course of processes of ovulation in experimental animals has been studied. The promising nature in this direction of the search for active analogs for luliberin has been shown.

In recent years, a large number of analogs of the releasing factor of the luteinizing hormone — luliberin — has been synthesized. The main efforts of research workers have been directed to obtaining effective inhibitors and compounds with a high agonistic activity. Such preparations can be used as contraceptive agents, and in the agricultural industry and clinical medicine in the treatment of certain endocrine and tumoral diseases [1, 2].

To obtain effective agonists, the glycine residue in the sixth position of the natural molecule is usually replaced by a D-amino acid residue and (or) the glycinamide residue in the tenth position by an ethylamide grouping [3]. The strategy for obtaining luliberin antagonists that has been developed amounts basically to the conversion into an inhibitor of a compound with a high agonistic activity by eliminating the histidine residue in the second position of the molecule or replacing it by a D-amino acid residue [4].

In our laboratory, certain new possible directions of the synthesis of active analogs of luliberin are being tested. One of such directions is the production of compounds with shortened amino acid sequences. The creation of active preparations of this type would make them more accessible for practical application and at the same time would permit new information to be obtained on the structure—activity interrelationships in the series of luliberin analogs.

The majority of the highly active analogs of the releasing hormone known at the present time contain from 8 to 11 amino acid residues in the molecule among which in many cases there are difficultly accessible unnatural amino acids such as, for example, substituted D-phenylalanine or amino acids causing certain complications in the course of peptide synthesis (such as histidine, tryptophan, and pyroglutamic acid) [5—8].

Repeated attempts to obtain luliberin analogs containing fewer than 8 amino acid residues have mainly led to inactive compounds as, for example, in the cases of H-Pro-

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