Acta Chemica Scandinavica B 33 (1979) 22—26

The Peroxidatic Oxidation of Some Phenolic Lignin Model Compounds

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The reactivity of some lignin model compounds, vanillyl alcohol, dehydrodivanillyl alcohol, isoeugenol, coniferyl alcohol, guaiacylglycerol-β-guaiacyl ether and dihydrosinapyl alcohol, as electron donors in the horseradish peroxidase-catalyzed reaction was studied by following the kinetics of Compound II of the peroxidase. Of the donors used, vanillyl alcohol and dehydrovanillyl alcohol are oxidized at a higher rate than the other lignin model compounds.

Addition of ethanol caused a large decrease in the reaction rate.

The enzymic oxidation and coupling of phenols is a subject of primary importance in the study of the biosynthesis and biodegradation of lignin. Recent evidence suggests that one of the enzymes involved in the biosynthesis of lignin is a peroxidase.[1] Horseradish peroxidase (HRP) has commonly been used for studying the reactions of lignin model compounds with peroxidases. HRP is a plant peroxidase of rather low substrate specificity, and a number of organic compounds serve as hydrogen donors in the peroxidatic oxidation reaction.

According to Chance [2] the reaction between peroxidase (E), hydrogen peroxide and a hydrogen donor (AH_2) can be represented as follows:

$$E + H_2O_2 \xrightarrow{k_1} \text{Compound I} \quad (1)$$

$$\text{Compound I} + AH_2 \xrightarrow{k_7} \text{Compound II} + AH\cdot \quad (2)$$

$$\text{Compound II} + AH_2 \xrightarrow{k_4} E + AH\cdot \quad (3)$$

where k_4 is the rate constant for the slowest, rate-determining step. In the presence of a donor k_7 is much greater than k_4 and cannot be measured for fast reacting phenols with the presently used mixing technique.[2]

The final stage in the biosynthesis of lignin is an oxidative polymerization of substituted *p*-hydroxycinnamyl alcohols.[3] The distribution of structural units in the polymer is determined by two main factors: (a) the rates of oxidation of the different phenols involved in the reaction and (b) the rates of different coupling modes, for instance β-β, β-O-4, β-5 (for terminology see Ref. 3). In the present work we are dealing with the first of these factors, the peroxidatic reaction rates of some representative lignin model compounds as catalyzed by horseradish peroxidase.

In many studies concerning the preparations of synthetic lignins (dehydrogenation polymer) the reactions are carried out in 50 % aqueous ethanol,[4] and in some cases different reaction products are encountered in aqueous solution and in 50 % ethanol. In order to test whether the addition of ethanol causes a change in the reaction mechanism a series of rate measurements was carried out in 50 % aqueous ethanol.

EXPERIMENTAL

Methods

Spectroscopy. Activity measurements with guaiacol were carried out with a Beckman DK-1A recording spectrophotometer. All other spectroscopic measurements were performed with a Cary 15 recording spectrophotometer with the cell compartment thermostated at 25 °C.

0302-4369/79/010022-05$02-50

The rate constant k_4 for the various hydrogen donors was determined by following the kinetics of Compound II [5] at 426 nm, where free peroxidase and Compound I are isosbestic.[6]

Materials

Horseradish peroxidase (donor:hydrogen peroxide oxidoreductase, EC 1.11.1.7) was purified as described by Paul and Stigbrand [7] and the slightly basic isoenzyme C2 [8] with an *RZ*-value of 2.5 was used in all the experiments. Control measurements using a peroxidase preparation with $RZ = 3.15$ gave the same results. The concentration of the enzyme was determined as pyridine ferrohemochrome according to Paul *et al.*[8] The activity of the peroxidase was checked with guaiacol using the conventional activity measurement of Chance and Maehly [10] with slight modifications.

Hydrogen peroxide solutions were prepared from "Perhydrol" (Merck), (30 % H_2O_2) and the concentration of the solution was determined both spectrophotometrically at 230 nm using $\varepsilon = 72.4$ M^{-1} cm^{-1} [11] and by iodometric titration.[12]

Ascorbic acid (pro analysi, Merck) solutions were freshly prepared in deaerated buffer solutions and were purged with nitrogen for 15 min immediately before use.

Guaiacol (rein, Merck) was used without further purification.

4-Hydroxy-3-methoxybenzyl alcohol, vanillyl alcohol, (*1*) was prepared by the sodium borohydride reduction of vanillin.[13]

2,2'-Dihydroxy-5,5'-di(hydroxymethyl)-3,3'-dimethoxybiphenyl, dehydrodivanillyl alcohol, (*2*) was prepared by the sodium borohydride reduction of dehydrodivanillin.[13]

(E)Isoeugenol, (*3*, $R = CH_3$). The (*E*) isomer was isolated from commercial isoeugenol (FLUKA) by recrystallization of the acetyl derivative.

1-(4-Hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol, guaiacylglycerol-β-guaiacyl ether, (*4*). The purified *erythro*-form was kindly supplied by Dr. Gerhard Miksche.[14]

3-(4-Hydroxy-3,5-dimethoxyphenyl)-1-propanol, dihydrosinapyl alcohol, (*5*) was prepared from ethyl hydrosinapate by reduction with lithium aluminium hydride. Yield 91 % m.p. 75 – 76 °C (Lit. m.p. 75.5 – 76.5).[15]

(E)-3-(4'-Hydroxy-3'-methoxyphenyl)prop-2-enol, coniferyl alcohol, (*3*, $R = CH_2OH$) was prepared by reduction of ethyl ferulate. Yield 50 %, m.p. 69 °C (Lit. m.p. 74 °C).[16]

RESULTS

Behaviour of the peroxidase in ethanol solutions. Using the classical activity measurement of HRP, it was found that the spectrum of the oxidation product of guaiacol in the wavelength range 350 – 550 nm is essentially independent of the presence of ethanol in the reaction medium (up to 50 % ethanol, v/v). On the other hand, the rate of the peroxidatic oxidation reaction is greatly diminished by the presence

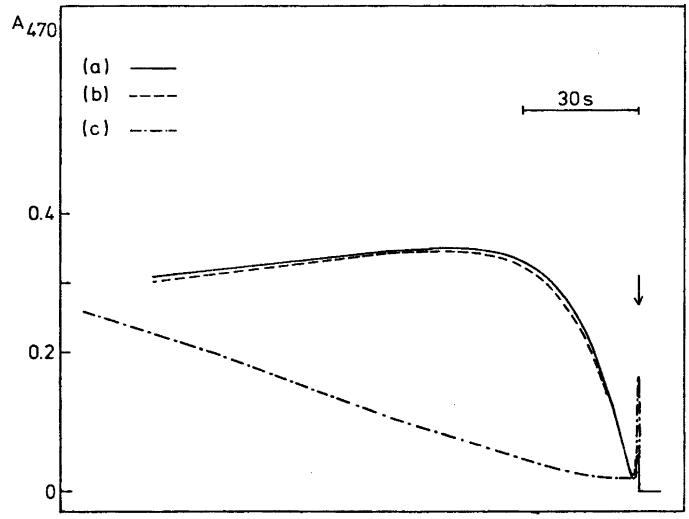


Fig. 1. Effect of ethanol on the rate of oxidation of guaiacol by horseradish peroxidase. (a) Reaction in buffer. (b) Reaction in buffer, enzyme diluted in ethanol. (c) Reaction in ethanol. Experimental details are described in the RESULTS-section.

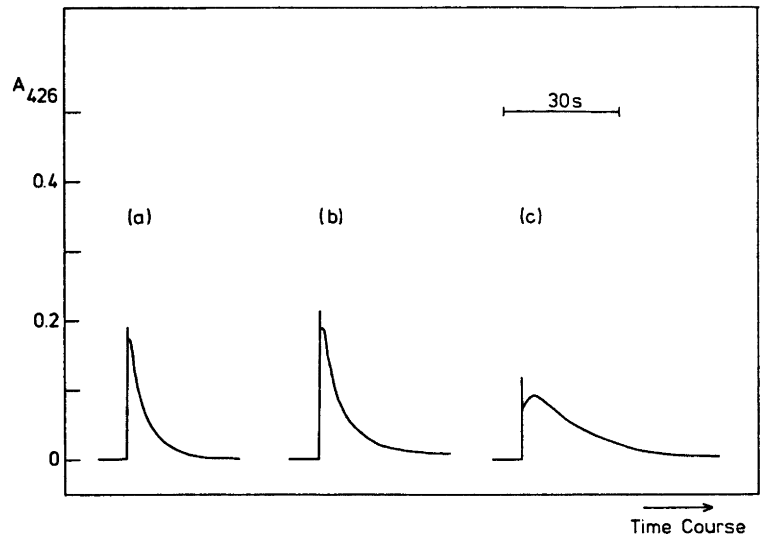
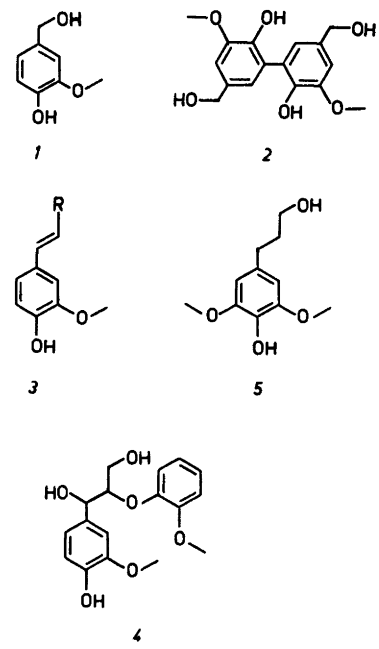


Fig. 2. Kinetics of formation and decomposition of Compound II upon addition of H_2O_2 to HRP in the presence of (a) vanillyl alcohol, (b) dehydrodivanillyl alcohol and (c) isoeugenol, respectively, as hydrogen donor.

of ethanol so that the rate is only about one tenth of the original value when the concentration of ethanol in the reaction mixture is raised from 0 to 50 %. The obvious explanation for the retarded rate was thought to be denaturation of the enzyme in the alcoholic medium. This, however, seems not to be the case. When 5 μl of 3.9 μM enzyme diluted in 50 % ethanol was added to the reaction mixture (2.0 ml) containing no ethanol (Fig. 1, b) the reaction did not differ markedly from that obtained when the enzyme was diluted in buffer (Fig. 1, a). Furthermore, the enzyme could be stored in 50 % ethanol at 4 °C for 24 h without loss of activity. Thus the retarded reaction rate in ethanol (Fig. 1, c) is not due to denaturation but rather depends on the dielectric properties of the medium. The native state of the enzyme in alcohol was also confirmed by recording the spectrum of HRP both in buffer and in 50 % ethanol solutions in the Soret-region, which is easily affected by denaturation. The spectra for HRP in the two media did not differ from each other.

Rate measurements. The reactivity of six lignin model compounds (Scheme 1) in the HRP-reaction was determined by measuring the rate constant k_4, which represents the conversion of Compound II to free peroxidase. For comparison, ascorbic acid was also used as electron donor for HRP. Fig. 2 shows a



Scheme 1.

Table 1. Rate constants ($k_4/10^3$ M^{-1} s^{-1}) for the reaction between horseradish peroxidase and various phenols. Measurements were made in 10 mM potassium phosphate buffer, pH 7.0 and in buffer containing 50 % (v/v) ethanol. The rate constants are mean values of at least three measurements. Ascorbic acid is included for comparison.

Electron donor	Buffer	Ethanol
Vanillyl alcohol (*1*)	99.5	1.8
Dehydrodivanillyl alcohol (*2*)	85.0	1.0
Isoeugenol (*3*, R = CH_3)	45.5	2.4
Coniferyl alcohol (*3*, R = CH_2OH)	12.2	[a]
Guaiacylglycerol-β-guaiacyl ether (*4*)	9.1	0.3
Dihydrosinapyl alcohol (*5*)	1.5	0.6
Ascorbic acid [b]	4.7	0.2

[a] Not determined. [b] Donor concentration 10 μm, others 3.9 – 5.3 μM.

reaction trace with vanillyl and dehydrodivanillyl alcohol as well as with isoeugenol. In the case of isoeugenol (Fig. 2, c) the concentration of Compound II does not reach its saturation value, which indicates that it is a poorer donor than the other two phenols. The rate constants of the various phenols are given in Table 1. The reaction in ethanol was usually rather slow and the rate constants calculated from such measurements can only be taken as estimates. This holds especially for guaiacylglycerol-β-guaiacyl ether (*4*) and dihydrosinapyl alcohol (*5*).

Dependence of the rate constant k_4 *on ethanol concentration.* As described above, the over-all reaction rate of guaiacol oxidation in the HRP-reaction is greatly affected by the presence of ethanol in the reaction medium. The rate constant k_4 for vanillyl and dehydrodivanillyl alcohol was determined in reaction mixtures that were 0, 10, 30 and 50 % (v/v) in ethanol. Fig. 3 shows the dependence of the rate constant k_4 on ethanol concentration. It can be seen that k_4 is radically reduced when the ethanol concentration goes up to 50 %, in agreement with the guaiacol experiments.

DISCUSSION

In the present work the kinetics of the formation and decomposition of Compound II were followed. Accordingly, only the fast enzymatic reaction is involved and the results describe the primary steps in the dehydrogenation process. The rate constants in Table 1 show that vanillyl alcohol (*1*) and dehydrodivanillyl alcohol (*2*) are oxidized at higher rates than the other lignin model compounds. Isoeugenol (*3*, R = CH_3) reacts rather rapidly, too. In contrast, coniferyl alcohol (*3*, R = CH_2OH) reacts more slowly than its structural analog, isoeugenol. Dihydrosinapyl alcohol is slowest of the donors used.

It is difficult to find a simple explanation for the different reaction rates obtained for these model compounds. The enhanced reactivity of vanillyl and dehydrodivanillyl alcohols may be due to their short sidechains, thus allowing binding close to the heme iron.[17] The notable difference in oxidation rates between isoeugenol and dihydrosinapyl alcohol is of interest because estimates of critical redox potential [18] and results from cross coupling experiments [19] imply that a 2,6-dimethoxy

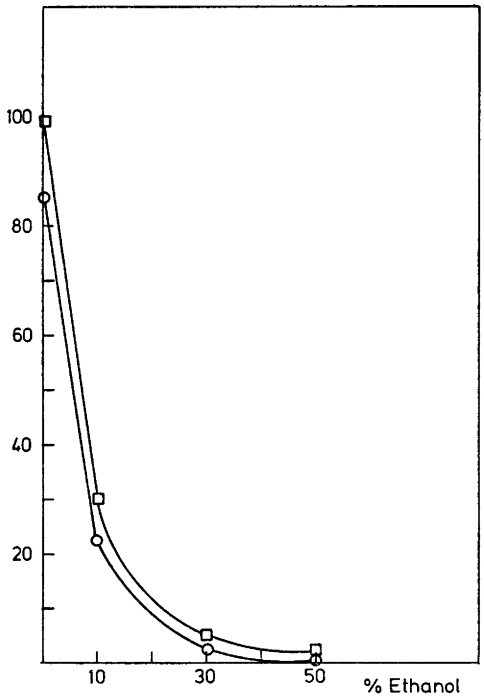


Fig. 3. Effect of ethanol concentration on the rate constant k_4 with vanillyl alcohol (□) and dehydrodivanillyl alcohol (○) as electron donor.

substituted phenol would be oxidized at a slightly higher rate than a 2-methoxy-4-vinyl substituted one.

It has earlier been assumed that the function of the enzyme is simply to catalyze the formation of aryloxy radicals.[20] Our results emphasize the importance of specific interactions between enzyme and substrate. Apparently the active site of the enzyme sets strict structural demands on the electron-donating substrate.

The addition of ethanol to the dehydrogenation mixture causes a large decrease in reaction rate but does not seem to cause any other change in the reaction mechanism. The decrease in the rate of peroxidase reactions in the presence of ethanol and other organic solvents has been interpreted in terms of the dielectric constant of the medium.[21] It has been suggested recently that the predominant effect of ethanol on peroxidase is to act as a ligand to the heme iron, and it thus operates as a competitive inhibitor.[22] Anyhow, perhaps due to a less concentrated enzyme solution, we did not observe the HRP–ethanol complex reported by Dunford and Hewson.[22]

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Received August 14, 1978.