OXIDATION OF LIGNINS AND THEIR COMPONENTS BY OXYGEN IN THE PRESENCE OF LACCASE FROM *Polyporus versicolor*. LIGNIN DETECTION BY AN ENZYME ELECTRODE

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The kinetics of the laccase-catalyzed oxidation of model lignin components by oxygen has been studied. It has been shown that in natural lignins the fragments containing guaiacyl groups are enzymatically oxidized without cleavage of the ether bond in the methoxy group. The enzyme has been immobilized by entrapping in polyacrylamide gel and also by covalent attachment to the surface of alkylamine glass. Immobilized laccase was used for the construction of sensors with the Clark oxygen electrode; such sensors can be employed for the determination of the content of lignin-like and phenolic compounds in waste waters.

Pollution of water reservoirs with waste water containing natural polymer lignin gives rise to secondary contamination by phenols resulting from the degradation of lignin. One of the aims of this investigation has been to develop a method of lignin analysis in waste waters and in natural water reservoirs. Electrochemical and spectral methods[1-3] are used at present for this purpose. Quantitative analysis of lignins by an enzyme electrode based on immobilized laccase seemed promising and a study of substrate specificity of laccase isolated from *fungi* was also considered of interest.

EXPERIMENTAL

Model lignin components were synthetized and purified in the laboratory of the Institute of Organic Chemistry, Academy of Sciences of the USSR. Lignins were obtained by the procedures described in the paper[4]. Laccase was prepared from the fungus *Polyporus versicolor* according to the method described[5]. The immobilization of laccase on acrylamide gel was effected with the reagents purchased from Reanal (Hungary). For the immobilization on alkylamine glass the Woodward's reagent from Sigma (U.S.A.) was used. The enzyme activity was assayed amperometrically using a Clark electrode (polarograph OH-105, Hungary).

Immobilization of laccase on polyacrylamide gel: In 1 ml of 0·1 mol l^{-1} acetate-phosphate buffer, 0·255 g of acrylamide, 0·0255 g of N,N′-methylenebisacrylamide, and 20 μl of 10% solution of N,N,N′,N′-tetramethylenediamine were dissolved at pH 5·0. The mixture was cooled down to 0°C and 0·2 ml of laccase solution containing 2 mg of protein and 2 mg of ammonium persulfate per ml was added with stirring.

Immobilization of laccase on alkylamine glass C-50: The Woodward's reagent (2 mg) was dissolved in 60 ml of distilled water and 60 μl of the enzyme solution (protein content 2 mg/ml) was added. The alkylamine glass C-50 (25 mg) was suspended in the solution with stirring. The suspension was set aside for 12 h at 4°C. The precipitate was centrifuged off. The supernatant was tested for enzymatic activity by the reaction with hydroquinone. The precipitate was washed twice with distilled water.

Enzyme electrode: The oxygen-type enzyme electrode was prepared by applying a layer of immobilized laccase on the teflon membrane of the Clark oxygen electrode. The immobilized laccase was fixed on the electrode with the aid of a dialysis membrane (laccase immobilized in polyacrylamide gel) or with a nylon net (covalent immobilization on alkylamine glass beads). The kinetic measurements were performed amperometrically in 0·05 mol l^{-1} acetate–phosphate buffer at pH 5·0 and 35°C.

RESULTS AND DISCUSSION

Lignins are believed to be natural polymers resulting from enzymatic dehydrogenation of unsaturated alcohols mainly containing the following groups: guaiacyl-(3-methoxy-4-hydroxyphenyl), veratryl(3,4-dimethoxyphenyl), syringyl(3,5-dimethoxy--4-hydroxyphenyl), and 4-hydroxyphenyl. In nature, lignins are bound to cellulose forming a lignocellulose complex. The exact mechanism of the complex degradation by individual factors is not yet known. Laccase (*p*-diphenol:O_2 oxidoreductase) contained in a variety of fungi including those destroying wood oxidizes various types of polyphenol compounds. To elucidate the mechanism of laccase-catalyzed lignin oxidation, model lignin components and the kinetics of their oxidation have been studied.

TABLE I

Kinetic parameters of oxidation of model lignin components by oxygen in the presence of laccase. Conditions: 0·05M acetate–phosphate buffer, pH 5·0, 35°C, $E_0 = 4{\cdot}1 \cdot 10^{-9}$ mol l^{-1}

Compound	V_{max}, mol l^{-1} min^{-1}	K_m, mol l^{-1}
Vanilic alcohol	$4{\cdot}6 \cdot 10^{-5}$	$4{\cdot}0 \cdot 10^{-4}$
Homovanilic acid	$4{\cdot}2 \cdot 10^{-5}$	$3{\cdot}5 \cdot 10^{-4}$
Vanilic acid	$4{\cdot}1 \cdot 10^{-5}$	$1{\cdot}0 \cdot 10^{-3}$
Dihydroeugenol	$4{\cdot}0 \cdot 10^{-5}$	$2{\cdot}6 \cdot 10^{-4}$
Eugenol	$3{\cdot}9 \cdot 10^{-5}$	$6{\cdot}0 \cdot 10^{-4}$
Vanillin	$2{\cdot}1 \cdot 10^{-5}$	$2{\cdot}0 \cdot 10^{-3}$
Guaiacol	$5{\cdot}0 \cdot 10^{-5}$	$3{\cdot}0 \cdot 10^{-4}$
Hydroquinone	$6{\cdot}0 \cdot 10^{-5}$	$4{\cdot}7 \cdot 10^{-4}$
Catechol	$5{\cdot}5 \cdot 10^{-5}$	$6{\cdot}0 \cdot 10^{-4}$

Table I presents the kinetic parameters of the laccase-catalyzed oxidation of model components by oxygen. It can be seen that these compounds can be divided into two groups with respect to the enzymatic reaction: some are laccase substrates and can be oxidized by oxygen, others on not participate in the reaction. The latter group consists of compounds containing veratryl and 4-hydroxyphenyl groups (*i.e.*, veratryl--propane-(1)-ol, veratryldehyde, veratrylglycerol, β-guaiacylglycerol ether, β-oxy-(O-methoxyphenyl)acetoveratrone, 4-hydroxyphenyl ethyl ketone, 4-hydroxy-benzal dehyde, and guaiacylpropane-3-ol). The former group is composed of guaiacyl-containing model lignin components. Table I shows that compounds of different structure which behave as enzyme substrates also show very similar values of maximal reaction rates and Michaelis constants. An exception represents guaiacyl-propane--3-ol which is not split off in the presence of laccase. The above data lead us to the conclusion that in natural lignins regardless of their origin only lignin fragments containing guaiacyl groups are oxidized enzymatically by laccase. It is the guaiacyl residue and not the side chains which is oxidized. Neither is cleaved the ether bond in the polymer molecule.

We plotted the calibration curves for vanillic alcohol, vanillin, vanillic and homovanillic acid, pyrocatechol, eugenol, dihydroeugenol, hydroquinone, and guaiacol obtained with the enzyme electrode containing laccase immobilized on polyacryl-

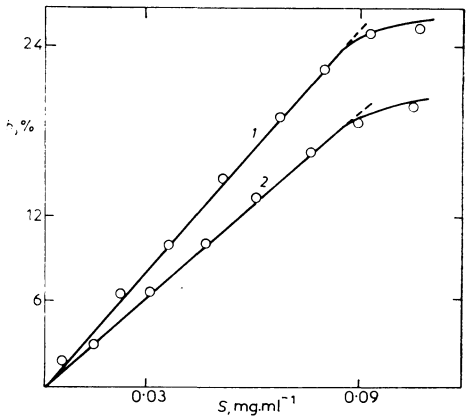

FIG. 1

Concentration dependence of the rate of pine lignin oxidation. Lignin solution in hot C_2H_5OH *1* and in dioxane *2*; *3* lignin residues insoluble in alcohol (solution in dioxane). Conditions: acetate–phosphate buffer, pH 5·0, 35°C, $E_0 = 4{\cdot}16 \cdot 10^{-9}$ mol l^{-1}

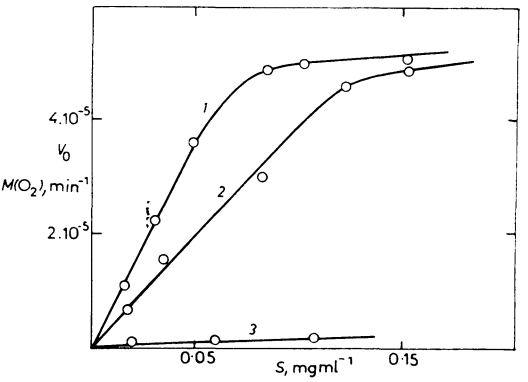

FIG. 2

Calibration curves for lignins. *1* Sulfate lignin; *2* alkaline sulfate lignin. Conditions: acetate–phosphate buffer, pH 5·0, 35°C

amide gel. The calibration curves were plotted from the steady state response of the electrode and coincided for the above substrates. The results obtained permit an enzyme electrode for the determination of lignin to be constructed. The lignins under study completely dissolve in dioxane and partially in ethyl alcohol. Fig. 1 shows the rate of oxidation of pine lignin isolated from sulfate liquor and measured in terms of absorption of oxygen as a function of lignin concentration. The maximum rates coincide for lignin dissolved in dioxane and alcohol. However, the concentrations at which no dependence of the rate of the enzymatic reaction on substrate concentration can be observed, are lower for lignin dissolved in alcohol than in dioxane. The lignin residue insoluble in alcohol is not oxidized by oxygen (curve 3). The V_{max}-value for the lignin samples studied is $(7 \pm 2)\ 10^{-2}$ mol O_2 min^{-1}. The IR spectra of lignin soluble and insoluble in ethyl alcohol differ a little, mainly in the region of the absorption of the carboxyl group (1 600 – 1 700 cm^{-1}). The results of the investigation of enzymatic oxidation of the model compounds allow us to postulate that the lignin residue insoluble in alcohol contains a small number of guaiacyl residues. Laccase immobilized in polyacrylamide gel was used in our initial experiment for the detection of lignins by an enzyme electrode. However, the high molecular weight of lignins prevents their diffusion into the polyacrylamide gel and no enzymatic reaction was observed. As a result there was no response from the electrochemical sensor in the presence of lignin. To eliminate the unfavorable effects caused by impossibility of diffusion into the gel layer, laccase was covalently immobilized on alkylamine glass C-50 beads; the Woodward's reagent was used as a crosslinking agent. The enzyme electrode was prepared by spreading a layer of alkylamine glass with immobilized laccase over a teflon membrane of the Clark oxygen electrode. The nylon net does not prevent lignin from diffusion to the enzyme. The enzymatic reaction brought about a decrease in oxygen concentration in the layer of immobilized enzyme thus changing the electrode response. It is convenient to express the electrode sensitivity (h) in this case as steady-state electrode response in per cent, of the whole scale of the instrument (250 mm). The calibration curves were measured with sulfate lignin and with alkaline sulfate lignin (Fig. 2). The calibration curves for lignins dissolved in dioxane and alcohol coincide. The time of the steady-state response to be set up is 5 – 6 min. The final response is a linear function of lignin concentration in the concentration range $4 \cdot 10^{-3} - 8 \cdot 10^{-2}$ mg/ml. When laccase is immobilized on alkylamine glass, 20 – 30% of the native laccase activity is retained. The stability of immobilized laccase does not differ essentially from that of the native enzyme. The method described permits a rapid quantitative analysis of lignin in solution to be performed.

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