

Carbohydrate-dependent Enzymic Quinone Reduction during Lignin Degradation

ULLA WESTERMARK and KARL-ERIK ERIKSSON

Swedish Forest Products Research Laboratory, Box 5604, S-114 86 Stockholm, Sweden

Indications for the existence of a new enzyme were obtained when *Polyporus versicolor*, a white-rot fungus, was grown on lignin agar plates supplemented individually with glucose, cellobiose or cellulose. Formation of chromophoric structures occurred through the action of a polyphenol oxidase (laccase) but was effectively reversed when cellulose was present in the agar plates. Under these conditions the fungus produced an extracellular enzyme which, in the presence of cellobiose, reduced the stable quinone tested, namely 3-methoxy-5-*tert*-butylbenzoquinone-(1,2). The enzyme together with cellobiose inhibited phenolic coupling of guaiacol by laccase and although oxygen consumption proceeded normally no polymeric products were formed.

It has long been known that the white-rot fungi readily degrade lignin in wood. Isolated lignin in submerged cultures seems, however, to be much more resistant to fungal attack. Under such conditions growth on lignin requires adaptation^{1,2} to the polymer and even then it is questionable if depolymerization occurs to any large extent.

It seems likely that submerged cultures do not offer the right growth conditions when lignin is the carbon source, maybe due to insufficient localization of the necessary levels of a combination of several enzymes required for depolymerization. We therefore investigated the use of a solid medium with lignin in which the fungus could produce high local concentrations of enzymes and, hopefully, degrade lignin. To achieve conditions as similar to wood as possible, we also incorporated cellulose in the growth medium to observe if this could affect the lignin degradation. These experiments have led to the discovery of an enzyme, cellobiose:

quinone oxidoreductase, which can reduce products of laccase oxidation and different quinones in a reaction in which cellobiose is required and simultaneously oxidized. The phenol-oxidizing enzyme laccase is induced in white-rot fungi both by lignin and simple phenols.³ The precise role of laccase during lignin degradation is not yet elucidated.

A recent review⁴ on lignin degradation concludes that whatever role phenol oxidases play, they can only be a part of the enzyme complex that catalyzes the complete decomposition of lignin.

This paper describes the experiments in which the quinone-reducing enzyme was found and the ability of the enzyme to stop phenolic coupling during laccase oxidation of guaiacol. An accompanying paper⁵ describes the induction and some properties of the enzyme.

MATERIALS AND METHODS

Organism. *Polyporus versicolor* L. ex Fr. isolate Mad. was obtained from Prof. E. B. Cowling, North Carolina State University, Raleigh, North Carolina, USA.

Substrate. The lignin preparation used was a kraft lignin, Indulin AT (Westvaco, Polychemical Div., Charleston, West Virginia, USA). The lignin was dialyzed against a large volume of distilled water (2 × 20 l per 5 g Indulin AT for 20 h) in a dialysis bag with a pore size of 24 Å to remove low molecular weight compounds and impurities. Phosphoric acid swollen cellulose⁶ was prepared from Munktell's cellulose powder No. 400.

Cultivation. A basal salt medium⁷ was used with the supplement of evenly suspended lignin and different carbon sources and with 1.5 % Noble agar (Difco, Detroit, Mich., USA). Lignin was dissolved in a minimal volume of 0.1 M

KOH and filter-sterilized. The pH of the complete medium was adjusted to 5.6 with phosphoric acid. The plates were incubated at 25°C and 90 % relative humidity.

Enzyme. Laccase ($O_2:p$ -diphenol oxidoreductase, E.C. 1.10.3.2) was obtained from *P. versicolor*.⁸ The enzyme solution was concentrated to approx. 1/40 of the original volume by ultrafiltration (Amicon UM 10) and the laccase was precipitated with solid ammonium sulfate (90 % of saturation at 25°C). The precipitate was dissolved in a minimum volume of distilled water, dialyzed and partially purified on a Sephadex G-75 chromatography column.

Cellulose-quinone oxidoreductase was prepared according to methods in the accompanying paper.⁵

Enzyme assays. Laccase activity was measured by following the production of colored material from guaiacol. The incubation mixture contained 25 μ mol guaiacol, 50 μ mol acetate buffer (pH 5.0) and the enzyme in a total volume of 5 ml. The color production was followed with a Klett colorimeter equipped with a blue filter ($\lambda = 400 - 465$) at 25°C.

The initial rate of formation of colored products was taken as a relative measurement of laccase activity.

Chemical determinations. Guaiacol was determined gas chromatographically with benzyl alcohol as an internal standard. Column: 10 % OV-1 on Chromosorb Q 100-120 mesh, 120°C. Retention times: guaiacol 8.9 min and benzyl alcohol 6.6 min.

Oxygen consumption was measured with an oxygen electrode (Rank Brothers, Bottisham, England) at 25°C.

Chemicals. The 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2) was kindly supplied by Dr. Augustine E. Opara of this laboratory.

RESULTS

Coloration of lignin agar plates by *P. versicolor*. *P. versicolor* was cultivated on agar plates containing 1 g/l of lignin individually supplemented with 3 g/l of glucose, cellobiose, or swollen cellulose as indicated. Fig. 1 shows plates (5 day cultivation time), where the surface mycelium has been removed. A few mm in front of the mycelium there was a reddening of the lignin in all plates. However, in those plates which contained cellobiose and cellulose this coloration started to disappear in the area in contact with the outermost edge of the mycelium and reappeared under the oldest mycelium in the center. The plates with lignin and lignin plus glucose had an even red color over the entire surface. The red color most likely originated from phenol oxidase oxidation of lignin or its

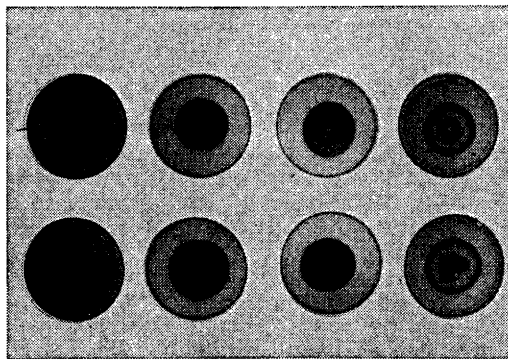


Fig. 1. *P. versicolor* grown on lignin agar plates supplemented with from left to right: nothing, glucose, cellobiose, and phosphoric acid swollen cellulose. The top row of plates shows the coloration caused by the fungus. The bottom row shows plates which have been flooded with guaiacol to stain for laccase activity.

degradation products. To test for laccase activity, the plates were flooded with a solution of 0.01 M guaiacol which gives an intense red to black color with laccase. The lower series of plates in Fig. 1 shows the plates after this staining. Laccase activity was found in all areas which were visibly red. The decolorized areas in plates with lignin and cellulose or cellobiose gave no laccase staining with guaiacol.

Extraction of enzymes from lignin-cellulose agar plates. *P. versicolor* was grown for 5 days on plates containing lignin and cellulose. The surface mycelium was then removed and the plates were separated into three zones according to Fig. 2. Equal amounts of the three zones were extracted with 0.1 M acetate buffer (pH

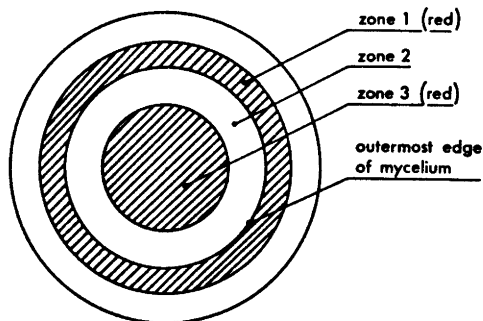


Fig. 2. Pattern of the lignin-cellulose agar plates used for extraction of enzymes from three zones.

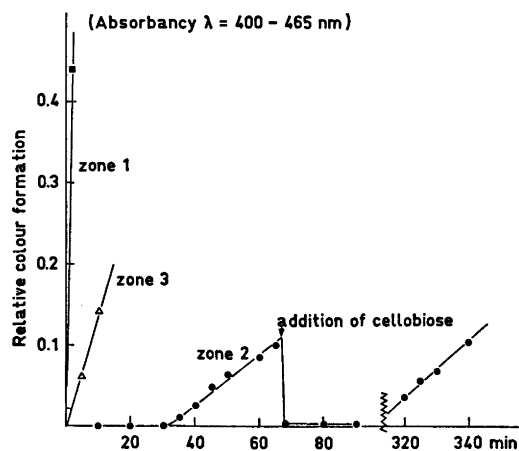


Fig. 3. Relative laccase activity in different zones of lignin - cellulose agar plates.

5.0) and centrifuged. Laccase activity was measured on the supernatants from the different zones. The results are shown in Fig. 3. The extract from zone 2 is of particular interest. Initially no color production from guaiacol was observed but after a while the color formation started and continued at a constant rate. If cellobiose was added, the color formation ceased and the colored products which had been formed disappeared. After a lag period depending upon the amount of cellobiose added, the formation of colored products was observed once more. It is obvious from these experiments that although laccase was present in zone 2, it did not react with guaiacol in the normal way. It is also clear that a reaction with cellobiose occurred in zone 2 and that this reaction inhibited the formation of colored products from laccase oxidation of guaiacol. If cellobiose was added to extracts from zone 3, a small decrease in the formation of colored products from guaiacol was found, but addition of cellobiose did not influence the laccase activity in extracts from zone 1.

Quinone reducing ability of the enzyme extracts from zone 2. The red products from laccase oxidation of both lignin or lignin degradation products and guaiacol are most likely due to quinoid structures.⁹ To determine if the extract from zone 2 could reduce quinones, a ten-fold concentrated extract was incubated with a stable quinone, 3-methoxy-5-*tert*-butyl-benzo-

quinone-(1,2). The quinone has an absorption maximum at 360 nm (acetate buffer, pH 5.0). The reduction of the quinone to the corresponding diphenol, was followed spectrophotometrically at this wave length. The enzyme extract from zone 2 could effectively reduce the quinone while boiled extract had no effect. It was also demonstrated that the reduction of the quinone was coupled to the oxidation of cellobiose. In an accompanying paper,⁵ some properties of the enzyme from *C. lignorum* is described, and from these results the name cellobiose:quinone oxidoreductase is suggested. It appears likely that the cellobiose:quinone oxidoreductase caused the disappearance of the red color in zone 2 and also prevented the formation of colored products from guaiacol in this zone. The enzyme cannot use glucose which would explain why no decolored zones occurred in plates with lignin and glucose. The width of the zones may reflect both the amount of enzymes induced and the concentration of the carbohydrate co-substrate.

Inhibition of phenolic coupling by the quinone reducing enzyme. To study the effect of cellobiose:quinone oxidoreductase on the laccase oxidation of guaiacol, the concentration of guaiacol was followed by gas chromatography and laccase activity measured by following oxygen uptake. The reaction mixture contained 25 μ mol guaiacol, 90 μ mol cellobiose, 1 mmol acetate buffer (pH 5.0), 25 μ mol benzyl alcohol (internal standard for gas liquid chromatography), laccase and cellobiose:quinone oxidoreductase in a total volume of 10 ml per sample. The control contained everything except cellobiose:quinone oxidoreductase. Samples and the controls were incubated at 25°C for different time periods after which the reaction mixture was extracted with 5 ml of ethyl acetate and the organic phase was analyzed for guaiacol and benzyl alcohol. Fig. 4 shows that all guaiacol in the control sample was polymerized in about 2 h. Under the same conditions less than 15 % of the guaiacol had been polymerized in the sample containing cellobiose:quinone oxidoreductase. Higher concentrations of oxidoreductase completely prevented the polymerization of guaiacol.

To determine if the laccase was inhibited by the solution of cellobiose:quinone oxidoreductase, the rate of oxygen consumption was

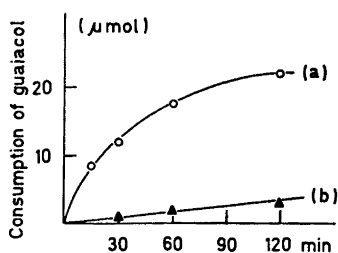


Fig. 4. Influence of cellobiose:quinone oxidoreductase on the consumption of guaiacol by laccase. The amount of guaiacol consumed is shown in (a) the control (laccase alone) and (b) sample containing both laccase and cellobiose:quinone oxidoreductase.

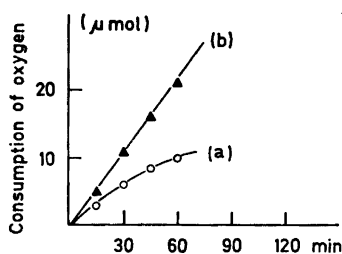


Fig. 5. Consumption of oxygen by laccase in the presence of cellobiose:quinone oxidoreductase. The samples are described in Fig. 4.

measured both in the sample and in the control. Fig. 5 shows that the initial rate of oxygen consumption was the same in both the sample and the control. The oxygen consumption in the control diminished with decreasing guaiacol concentration. In the sample containing cellobiose:quinone oxidoreductase the rate of oxygen consumption was constant. The oxygen consumption before addition of laccase was about the same for both the sample and the control and was subtracted from the readings. It is known that the preliminary product of laccase oxidation of phenols is the aryloxy radical and that the subsequent phenolic coupling is non-enzymatic.¹⁰ Thus, the laccase as usual formed the guaiacoxy radicals but, instead of coupling, the radicals were reduced back to the phenolic state by the cellobiose:quinone oxidoreductase.

It is very likely that this is the same enzyme which reacts with quinones and with the laccase oxidation products. As described in the ac-

companying paper both of these hydrogen acceptors give the same oxidation product from cellobiose. Preliminary enzyme purification experiments with an ion exchanger and isoelectric focusing have not separated the activities.

DISCUSSION

The wood-degrading fungi are very specialized in the utilization of their substrate wood. In most cases, the white-rot fungi remove lignin and cellulose or hemicellulose simultaneously, perhaps implying that the enzymic degradation of the different wood components is linked. The cellobiose:quinone oxidoreductase requires both a carbohydrate and an oxidized phenol for its activity and both of these substrates are modified during the reaction. Thus, a study of the degradation of pure cellulose and pure lignin would probably not give the same products as from the degradation in wood.

When fungi are cultivated on lignin with or without added glucose they excrete large amounts of phenol oxidases which cause a polymerization of phenolic substances. The results in this paper show that in a more wood-like surrounding, quite different reactions can occur. The laccase functions as normal, but its initial products are effectively reduced back to the phenolic state and polymerization is prevented.

The ability of white-rot fungi to reduce quinones has been observed earlier.¹¹ In this case the white-rot fungus *Polystictus sanguineus* was cultivated on agar plates containing small amounts of phenols. The phenols were first oxidized to colored products (probably quinones) but this color began to disappear after ten or twelve days. Agar plates containing preformed quinones were also slowly decolorized. It was suggested that this color change was due to a quinone reduction process.

Protocatechuate-3,4-oxygenase and catechol-1,2-oxygenase are the key enzymes in the catabolism of aromatic substances in most fungi.¹² It is known that these enzymes are inhibited by *o*- and *p*-benzoquinone.¹³ If the lignin is degraded to simple phenols or quinones, it must be of importance for the fungus to keep the phenols in a reduced state to ensure the further breakdown of the aromatic nucleus. Cellobiose:quinone oxidoreductase can obviously serve this function.

Acknowledgement. We wish to thank Majlis Jansson for skilful technical assistance and Dr. A. Opara for supplying the quinone. We are also indebted to Dr. B. Lindgren and Dr. J. Gierer of this laboratory for stimulating discussions.

REFERENCES

1. Day, W. C., Pelczar, Jr., M. J. and Gottlieb, S. *Arch. Biochem.* 23 (1949) 360.
2. Ishikawa, H., Schubert, W. J. and Nord, F. *Arch. Biochem. Biophys.* 100 (1963) 131.
3. Grabbe, K., Koenig, R. and Haider, K. *Arch. Mikrobiol.* 63 (1968) 133.
4. Kirk, T. K. *Ann. Rev. Phytopathol.* 9 (1971) 185.
5. Westermark, U. and Eriksson, K.-E. *Acta Chem. Scand. B* 28 (1974) 209.
6. Walseth, C. S. *Tappi* 35 (1952) 228.
7. Kirk, T. K. and Kelman, A. *Phytopathology* 55 (1965) 739.
8. Fåhraeus, G. and Reinhammar, B. *Acta Chem. Scand.* 21 (1967) 2367.
9. Lindgren, B. O. *Acta Chem. Scand.* 14 (1960) 2089.
10. Brown, B. R. In Taylor, W. I. and Battersby, A. R. *Oxidative Coupling of Phenols*, Dekker, New York 1967, p. 167.
11. Law, K. *Physiol. Plantarum* 12 (1959) 854.
12. Cain, R. B., Bilton, R. F. and Darrah, J. A. *Biochem. J.* 108 (1968) 797.
13. Bilton, R. F. and Cain, R. B. *Biochem. J.* 103 (1968) 829.

Received September 26, 1973.