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COOPERATION BETWEEN FUNGAL LACCASE AND GLUCOSE OXIDASE IN THE DEGRADATION OF LIGNIN DERIVATIVES

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Key Word Index—*Trametes versicolor*; fungus; enzyme cooperation; laccase; glucose oxidase; lignosulphonates; lignin depolymerization.

Abstract—In the presence of *Trametes versicolor* laccase, generation of quinonoid intermediates formed from a high-molecular-weight fraction of lignosulphonates (Peritan Na) was observed. The addition of glucose oxidase caused a diminution of the quinone level; thioglycolic acid intensified this process. When both laccase and glucose oxidase were incubated with the high-molecular-weight fraction, depolymerization was more extensive than in the experiment omitting glucose oxidase. In the case of the low-molecular-weight fraction, these two enzymes operated in concert and the polymerization process was disturbed due to glucose oxidase activity. Therefore the action of glucose oxidase in reducing quinones improved the efficiency of lignin depolymerization.

INTRODUCTION

The biological transformation of lignin is one of the most important processes occurring in the biosphere. However, the pathways of its degradation have not been elucidated and little is known about the enzymes involved.

Our previous studies [1] suggest that fungal laccase (EC 1.10.3.2) is able to depolymerize soluble lignin (Peritan Na). Simultaneous polymerization and depolymerization were observed; however, the former was predominant due to the repolymerization of low-molecular-weight products [2].

On the other hand, the decomposition of lignin in growing fungal culture is very extensive. Numerous fungi able to degrade this polymer excrete laccase, e.g. *Trametes versicolor*, *Pleurotus ostreatus*, *Sporotrichum pulverulentum* and *Fomes annosus* [3–7]. Some of these species also possess glucose oxidase activity [7]. Since glucose oxidase can reduce quinones to phenols [7, 8], it could also affect quinonoid intermediates appearing as a result of laccase action on lignin. Consequently, there is a possibility of cooperation between these enzymes in lignin degradation. The aim of our present work was to test whether glucose oxidase operates in concert with laccase to bring about the decomposition of high-molecular-weight lignin.

RESULTS AND DISCUSSION

Figure 1 demonstrates the generation of quinonoid intermediates formed from fraction I of Peritan Na in the presence of laccase. An absorption maximum between 380 and 400 nm appeared; the absorbance was directly proportional to the time of incubation. Similar results were obtained by Green [7], who investigated the generation of quinonoid intermediates *in situ* from chlorogenic acid by *T. versicolor* laccase. However, in our experiments, after longer incubation of fraction I with the enzyme, the 380 nm absorption peak became constant and the quinone level did not increase until successive amounts of laccase were added (Fig. 2). This may suggest inhibition of laccase by the products formed.

The addition of glucose oxidase to the reaction mixture caused a quantitative decrease of quinonoid intermediates generated by laccase and there was a new lower quinone level (Fig. 3). This may result from the glucose oxidase activity, which effects transformation of quinones to phenolic compounds [7]. Diminution in quinone concentrations was more remarkable when thioglycolic acid, a specific laccase inhibitor, was introduced to the mixture (Fig. 3). In this case, blocking of further formation of quinonoid intermediates occurred.

The effect of glucose oxidase on quinonoid inter-

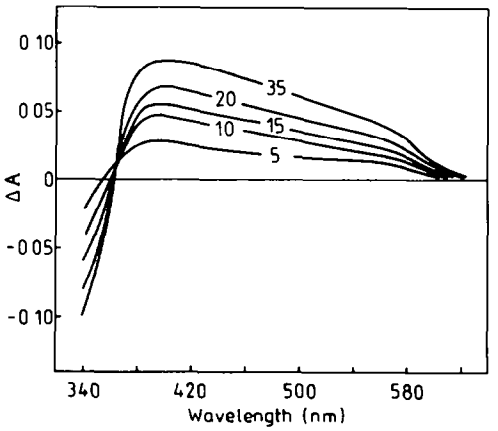

Fig. 1. Generation of quinonoid intermediates *in situ* from fraction I of Peritan Na by *T. versicolor* laccase. Visible spectra were measured at 5, 10, 15, 20 and 35 min after addition of enzyme.

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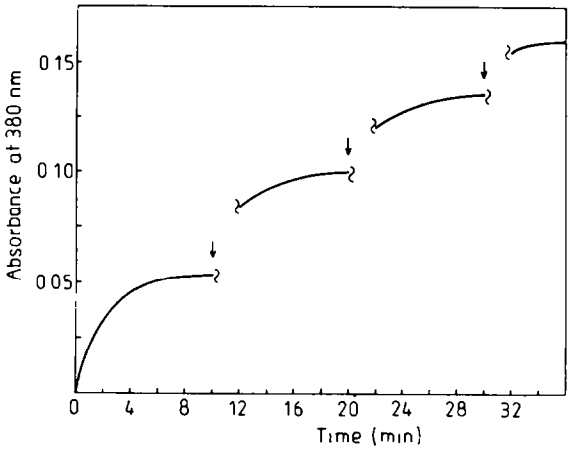

Fig. 2. Influence of laccase activity on generation of quinonoid intermediates from the high-molecular-weight fraction I of Peritan Na. At $t = 0$, 1.33 units of laccase were added and quinone appearance was monitored with time at 380 nm. Successive amounts of laccase (1.33 units each time) were introduced after 10, 20 and 30 min, as indicated by arrows.

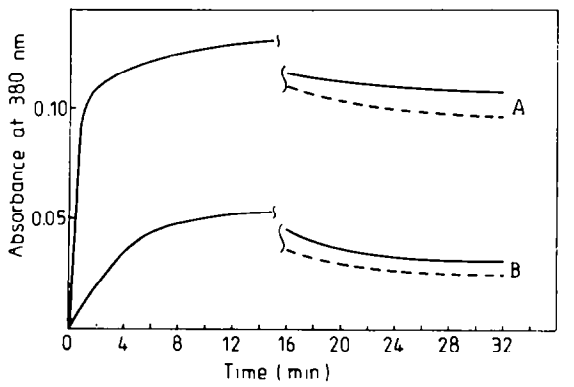

Fig. 3. Effect of glucose oxidase on the reduction of quinones formed from the high-molecular-weight fraction I of Peritan Na in the presence of laccase. Laccase was used in the amounts of 4 units (A) or 1 unit (B). After 15 min of incubation, glucose oxidase (40 units) was added. The same procedure was repeated with 0.5 mM thioglycolic acid introduced to the mixture after 15 min incubation with laccase (indicated by dashed lines).

mediates was studied by Green [7], who obtained similar results using chlorogenic acid as substrate. According to this author and also to Alberti and Klibanov [8], glucose oxidase is able to reduce quinones to hydroquinones when its second substrate, oxygen, is replaced by another electron acceptor, namely quinone. The enzyme can operate in a laccase–glucose: quinone oxidoreductase cycle, which provides reduced phenolic compounds [7]. This may be significant in the process of lignin degradation.

When laccase and glucose oxidase were incubated with fraction I, depolymerization occurred (Fig. 4). The quantities of low M_r products were larger than those in the experiment omitting glucose oxidase. Moreover, displacement of the peaks towards the lower M_r region of the chromatogram was noticed (Fig. 4).

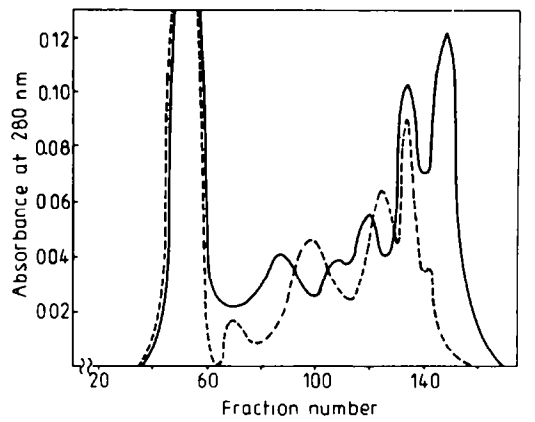

Fig. 4. Elution pattern of the high-molecular-weight fraction I of Peritan Na on a Sephadex G-50 'superfine' column (185 × 2 cm) after incubation with both laccase and glucose oxidase. Flow rate = 1 ml/min. The control (omitting glucose and glucose oxidase) is indicated by a dashed line.

In the case of the low M_r fraction IV, a different process occurred. The presence of both laccase and glucose oxidase disturbed polymerization and the high M_r maximum was smaller in comparison with that obtained when laccase acted alone (Fig. 5).

These results support the hypothesis of cooperation between these two enzymes, as proposed by Green [7]. They also confirm our earlier suppositions concerning the capacity of laccase to bring about depolymerization [1]. When both laccase and glucose oxidase were present, depolymerization was greatly enhanced. Under these aerobic conditions, glucose oxidase preferentially utilizes quinonoid oxygen, which is more active than molecular O_2. Quinonoid radicals are generated by the laccase and their immediate reduction prevents their repolymerization as well as their inhibition of laccase (Fig. 2).

This enzyme has been known to possess demethylating activity towards both lignin subunits [9–13] and lignin preparations [14–16], which was shown either by methanol release [11–13, 15, 16] or by methoxyl group diminution [9, 14]. During demethylation, laccase acts on

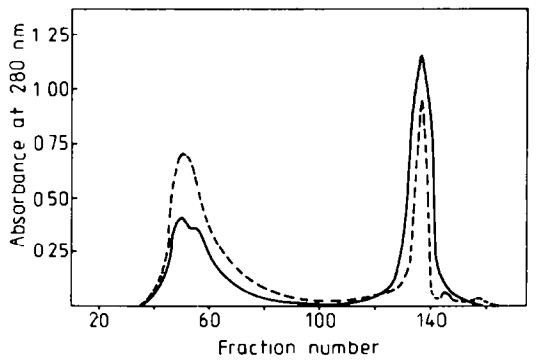

Fig. 5. Elution profile of the low-molecular-weight fraction IV on a Sephadex G-50 'superfine' column (185 × 2 cm) after incubation with both laccase and glucose oxidase. Flow rate = 1 ml/min. The control (omitting glucose and glucose oxidase) is indicated by a dashed line.

aryl–O–alkyl C–O bonds. If the enzyme is able to attack this kind of bond, it would possibly cleave other C–O–C bonds commonly found in lignin polymers, e.g. aryl–O–aryl ether bonds linking phenylpropane units. This process seems to be of basic significance in lignin degradation. It may also explain the depolymerization of Peritan Na observed in our previous studies [1]. According to the scheme of Harkin and Obst [10] and Ishihara and Miyazaki [11], demethylation caused by laccase is connected with oxidation. However, the oxidation process results in subsequent polymerization, when it goes too far. Radicals formed after demethylation and oxidation may react with each other, but the presence of glucose oxidase, which can reduce them to phenols, prevents repolymerization. Consequently, glucose oxidase hinders spontaneous repolymerization of quinonoid intermediates produced by laccase. Thus the efficiency of depolymerization is greatly improved. The same mechanism probably exists in nature, when various enzymes excreted by the fungus may operate in concert to transform lignin or lignocellulose into effectively utilized carbon sources.

EXPERIMENTAL

Chemicals. The commercial sodium lignosulphonates of spruce wood origin (Peritan Na) were obtained from Norcem As. (Oslo, Norway); syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehydazine) was from Aldrich. Glucose oxidase from *A. niger*, Type II, was purchased from Sigma.

Culture conditions. Trametes versicolor (L. *ex* Fr.) Pil, No. 7 was obtained from the Department of Biochemistry, University of M. Curie-Skłodowska, Lublin. The culture was grown for 3 weeks in stationary conditions at 24° in 1.5 l. flasks. Each flask contained 150 ml of a medium prepared as in ref. [1]. At the final step of cultivation, laccase production was promoted by xylidine and after 20 hr of incubation the enzyme was isolated [1].

Laccase assay. The isolation of extracellular laccase was performed directly as in ref. [1]. Protein was determined by the method of Lowry *et al.* [17]. Laccase activity was examined by the syringaldazine method [18], but instead of MES buffer 0.1 M citrate–phosphate buffer, pH 5, was applied. The activity was expressed in international units, as described in ref. [18].

Fractionation of Peritan Na. Peritan Na was fractionated on a Sephadex G-50 'superfine' column (185 × 2 cm), as in ref. [1]. Fraction No. I (M_r *ca* 97 000) and fraction No. IV (M_r *ca* 1000) were used in further expts.

Assay of quinonoid intermediates. To 2 ml fraction I (2 mg in 0.1 M citrate–phosphate buffer, pH 5) 1 unit of laccase was added and the visible spectrum was scanned on a spectrophotometer against the control prepared similarly but containing the boiled enzyme. Continuous scans were measured at various time intervals after enzyme addition, as in ref. [7].

Changes in the amount of quinones were also measured when successive amounts of laccase were added in the conditions described above, and monitored with time at 380 nm. Similar expts were performed with 20 units [7] of glucose oxidase added to the reaction mixture after 15 min incubation with laccase alone. In this case, 3.3 mM glucose was also included [7]. The control contained the boiled enzymes. The same procedure was repeated with 0.5 mM thioglycolic acid added just before introducing glucose oxidase.

Gel permeation chromatography. All expts were performed under aerobic conditions. Fraction I (5 mg), laccase (2.5 units) and glucose oxidase (100 units) were incubated at 20° in 5 ml 0.1 M citrate–phosphate buffer, pH 5, containing 3.3 mM glucose. After 1 hr the reaction was stopped by acidifying to pH 1 with 6 M HCl. To analyse the products, the reaction mixture was applied onto a Sephadex G-50 'superfine' column (185 × 2 cm) and then eluted and detected as in ref. [1]. The same procedure but without glucose oxidase and glucose was repeated as a control.

Similar experiments were also performed with fraction IV, but using 1 unit of laccase.

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