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THE EFFECT OF FUNGAL LACCASE ON FRACTIONATED LIGNOSULPHONATES (PERITAN Na)

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Key Word Index—*Trametes versicolor*; fungus; laccase; lignosulphonates; lignin depolymerization; lignin polymerization.

Abstract—Two fractions obtained after chromatography of lignosulphonates on Sephadex G-50, varying in M_rs, were treated with extracellular *Trametes versicolor* laccase. After incubation of the low M_r fraction, polymerization was observed, while in the case of the high M_r fraction the reverse process occurred. As a result of depolymerization, five new lower M_r fractions appeared. The reaction reached peak level after 2 hr of incubation and then the quantities of the products diminished, possibly due to their repolymerization. These studies indicate that laccase possesses both polymerization and depolymerization activity though the former was predominant.

INTRODUCTION

Laccase (EC 1.10.3.2) is a multiple form enzyme excreted into medium by mycelia of several Basidiomycetes, Ascomycetes and Deuteromycetes. In some fungi, laccase can be induced by xylidine or methoxyphenolic acids [1–5].

The enzyme was supposed to take part in the transformation of lignin, being involved in the process of demethylation [6–11]. Kirk *et al.* [12], using syringyl glycol-β-quaiacyl ether as a model lignin compound, demonstrated that laccase is able to cleave an etheric bond of the substrate. The depolymerization of the Björkman lignin macromolecule was described by Ishihara and Miyazaki [13], who isolated 2,6-dimethoxy-*p*-benzoquinone from the degradation products that appeared after laccase action. Furthermore, Ander and Eriksson [14] reported that the decomposition of lignin is closely connected with the presence of the enzyme in fungal culture. On the other hand, laccase has long been known to catalyse the polymerization of coniferyl alcohol providing its oxygenation to semi-quinone radicals which link together spontaneously, yielding molecules akin to native lignin [15, 16]. The results of later studies by Hüttermann *et al.* [17] and Haars and Hüttermann [18] concerning the activity of laccase towards either lignin subunits or its polymer confirm the above findings. These authors are convinced that the enzyme causes polymerization rather than degradation of lignin.

In order to elucidate the problem whether laccase is able to polymerize or depolymerize lignin, we have examined the activity of the highly purified enzyme towards various fractions of lignosulphonates (Peritan Na) which constitute an adequate model of native lignin.

RESULTS AND DISCUSSION

The fractionation of Peritan Na on Sephadex G-50 column is shown in Fig. 1. The approximate M_rs of the fractions were: I—97 000; II—8000; III—4300; IV—1000.

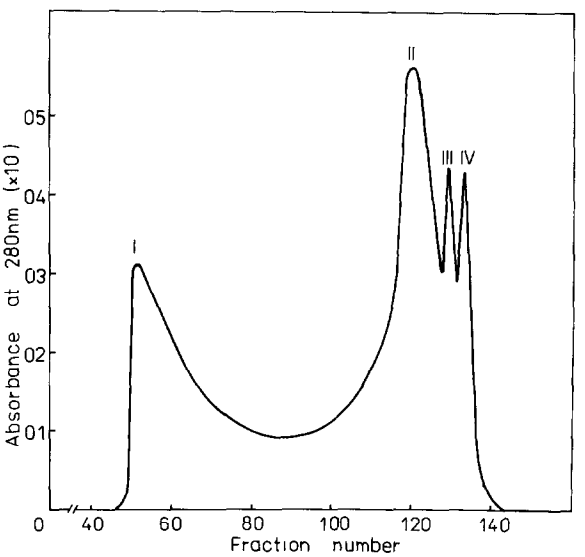

Fig. 1. Fractionation of Peritan Na on a Sephadex G-50 'fine' column (185 × 2 cm). (I–IV) Numbers of fractions.

For further experiments we chose only two fractions: high-M_r I and low-M_r IV.

The activity of laccase towards both fractions is indicated by the formation of colours in the reaction mixtures. Both substrates formed dark-yellow opalescent products, but the low-M_r fraction caused more brownish coloration. Gel filtration chromatography of fraction IV after incubation with laccase is shown in Fig. 2. After reaction for 1 hr the enzyme caused the lignosulphonate to polymerize. This is shown by the appearance of the exclusion peak at the high-M_r region of the chromatogram. The average M_r of the new fraction was close to that of fraction I, i.e. 97 000.

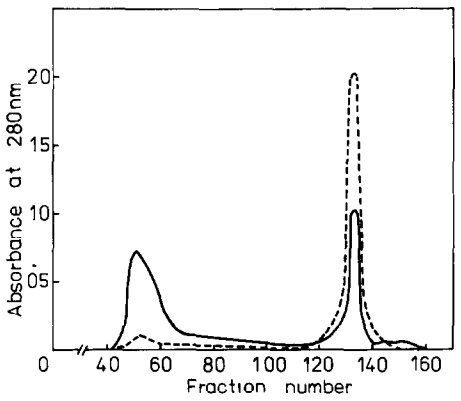

Fig. 2. Elution profile of fraction IV on a Sephadex G-50 'fine' column (185 × 2 cm) after incubation with laccase for 1 hr. The control is marked by a dashed line.

In contrast, when the high-M_r fraction I was treated with laccase, a depolymerization effect was observed (Fig. 3). As a result of the enzyme activity, five new lower M_r fractions were found with the following approximate M_rs: 1—74000; 2—9000; 3—1800; 4—1000; 5—850. It must be said that the M_r of the new fraction 4 is close to that of fraction IV obtained during fractionation of Peritan Na. Studying the depolymerization process further, we examined the dynamics of the appearance of low-M_r fractions. Figure 4 illustrates the changes in the amounts of new products connected with the time of incubation. The optimal time for accumulation of all new fractions was about 2 hr (Fig. 3A), and when it was longer, the quantities of products diminished (Fig. 3B).

These results show that laccase possesses both polymerization and depolymerization activity. The former is, however, dominant; this can be seen when the elution profiles shown in Figs. 2 and 3 are compared. The quantities of the depolymerization products are lower than the polymerization ones and vary in time. As was mentioned above, they decrease after a longer incubation with laccase.

Our conclusions refer to the different opinions of the two groups of authors cited earlier in the paper, i.e. the followers [12, 14] and the opponents [17, 18] of the hypothesis that laccase causes depolymerization of lignin. It is worth saying that those authors who deny the possibility of depolymerization, draw their conclusions from experiments done *in vivo*, where changes in the M_rs of lignins during the growth of fungus were observed. In these studies the activity of laccase was exposed by use of inhibitors, the specificity of which is not clear.

In our assays, the pure laccase preparation isolated earlier from fungal culture was examined. The results mainly relate to those of Ishihara and Miyazaki [13] who found the double function of the enzyme and showed that polymerization dominates over depolymerization. This is also in agreement with the effect noticed by Kaplan [19]. He suggested that depolymerization cannot proceed to the point of yielding considerable quantities of low M_r compounds because they are repolymerized quickly. It may explain the relatively small amounts of products formed during incubation of fraction I with laccase (Fig. 3).

Wojtaś-Wasilewska *et al.* [20] investigated changes in the M_rs of lignosulphonates during the growth of *Pleurotus ostreatus* culture. They observed both degradation and polymerization, and noticed that the intensity of the transformation was inversely proportional to the M_r of the fraction studied. Simultaneous polymerization and depolymerization caused by a pure enzyme was found by Łobarzewski *et al.* [21]. Peroxidase, which had earlier been isolated from the fungal material and immobilized, catalysed both changes in the M_rs of lignosulphonates.

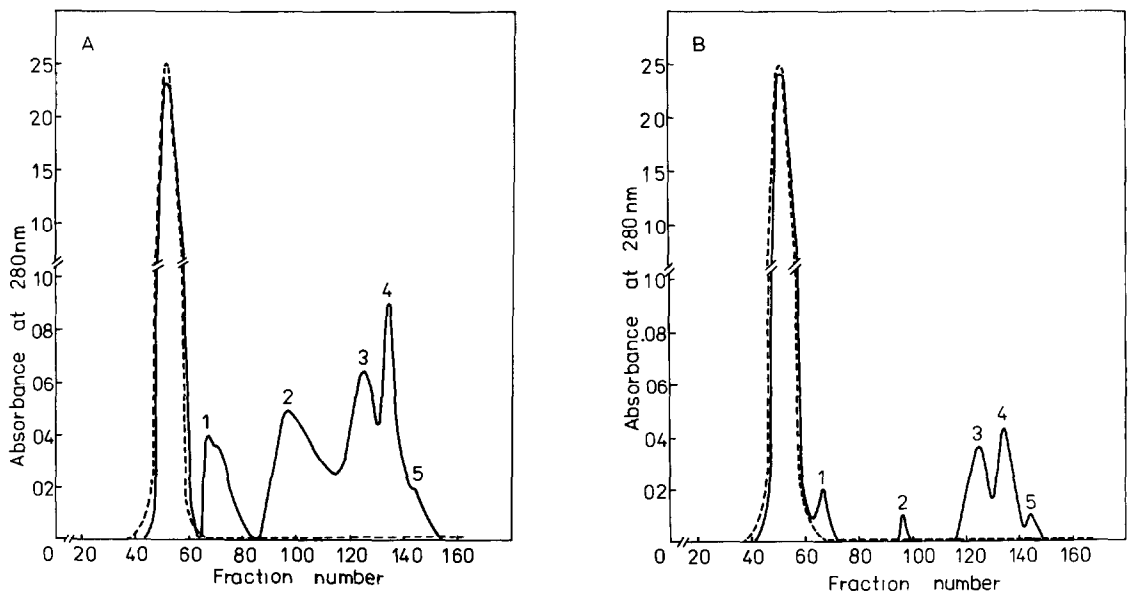

Fig. 3. Elution profiles of fraction I on a Sephadex G-50 'fine' column after incubation with laccase. (A) 2 hr, (B) 8 hr of incubation. Controls are marked by dashed lines.

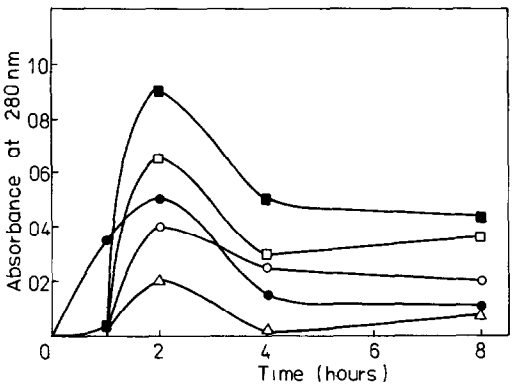

Fig. 4. The dynamics of the appearance of new fractions formed during incubation of fraction I with laccase. (○——○) Fraction 1; (●——●) fraction 2; (□——□) fraction 3; (■——■) fraction 4; (△——△) fraction 5.

In a further paper, Lobarzewski [22] studied the effect of immobilized peroxidase-containing fungal mycelium fragments of *Trametes versicolor* on Peritan Na. He observed the release of free phenolic compounds during the treatment of Peritan Na, dependent upon pH, temperature and reaction time.

The studies of the authors mentioned above support our results and we may conclude that the appearance of either polymerization or depolymerization possibly depends on the size of the molecule. When low-M_r lignin was a substrate of laccase, polymerization was observed, while the opposite process occurred in the case of high-M_r lignin (Figs. 2 and 3).

Ishihara and Miyazaki [13] suggested that the polymerization and depolymerization activities of laccase can result from the oxidative ability of the enzyme towards phenols or polyphenols, and radicals formed react with each other causing both processes. Such phenolic substrates could be produced in the course of demethylation. This function, described also for laccase [6–11], seems to be the first step of lignin biotransformation [23].

Since in nature lignin is closely connected with cellulose, forming a lignocellulose complex, many different enzymes are likely to participate in its degradation. Laccase is assumed to co-operate with some of them, e.g. cellobiose: quinone oxidoreductase [24] or glucose oxidase [25]. However, further studies are needed to clarify this problem.

EXPERIMENTAL

Chemicals. The commercial Na lignosulphonates of spruce wood origin (Peritan Na) were obtained from Norcem As., Oslo, Norway. Syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehydazine) was from Aldrich.

Culture conditions. Trametes versicolor (L. ex Fr.) Pil. No. 7 was obtained from the Department of Biochemistry, University of Lublin. The organism was kept on agar slants according to ref. [6]. The culture soln was based on Czapek Dox and Lindeberg media containing in 1 l. H_2O, 30 g sucrose, 10 g glucose, 2.5 g asparagine, 0.5 g KCl, 0.45 g KH_2PO_4, 0.17 g Na_2HPO_4, 3 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$, 50 mg $CaCO_3$, 9.7 mg $MnCl_2$, 5 mg $FeCl_3 \cdot 6H_2O$, 2 mg $ZnCl_2$, 4 mg $CuSO_4 \cdot 5H_2O$, 2 mg $Na_2B_4O_7$, 4 mg $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg thiamine; pH 5.5. The 150 ml cultures were grown for 3 weeks in 1.5 l. Roux flasks at 24° under stationary conditions. At the final step of cultivation, laccase production was promoted by xylidine as an inducer according to ref. [26], and the enzyme was isolated after 20 hr of incubation.

Isolation of laccase. The extracellular enzyme was isolated from the culture filtrate directly to the method of ref. [4]. The mycelium was separated from the culture by filtration through a Büchner funnel, and the protein-containing filtrate was treated by 3 vol. of cold Me_2CO to precipitate the enzyme. After centrifugation (10 min at 21 000 *g*) and dissolving the ppt. in 0.1 M NaPi buffer (pH 6), the further procedure, i.e. column chromatography on Sephadex G-50 and DEAE-Sephadex A-50, was performed as described in ref. [4].

The final laccase-rich fractions eluted from the DEAE-Sephadex A-50 column were concd to *ca* 10-fold by ultrafiltration through a Diaflo YM membrane (Amicon) according to ref. [27].

Protein and laccase determination. The protein content was determined according to ref. [28]. Laccase activity was examined directly using the syringaldazine method described in ref. [29] but instead of MES buffer 0.1 M citrate–Pi buffer was applied. One unit of laccase was defined as that amount which at pH 4.5 caused a change in *A* of 1 unit/min at 525 nm in 3.5 ml 0.1 M citrate–Pi buffer containing 0.3 ml 0 1 mM (150 pmol) syringaldazine in EtOH at 20°.

Fractionation of Peritan Na *by molecular filtration on Sephadex G-50.* The preparation of Peritan Na was separated into four fractions according to the method of ref. [20], where the industrial Na lignosulphonates were fractionated. In our experiments we applied a commercial preparation of Peritan Na. For prep. gel chromatography, a Sephadex G-50 'fine' column (185 × 2 cm) was used. Each time, 75 mg of the preparation dissolved in 1.5 ml H_2O was fractionated. The samples (3 ml) were eluted with H_2O (flow rate 0.5 ml/min) and the A_{280} was read against H_2O. Four fractions corresponding to the four peaks in the elution profile were collected and concd under the red. pres. and then lyophilized. The approximate M_rs of the fractions were determined by gel-permeation chromatography according to ref. [21].

Analytical chromatography was performed similarly but instead of a H_2O soln, 5 mg Peritan Na or its fraction in 5 ml 0.1 M citrate–Pi buffer (pH 4.5) solution was applied onto the column.

Incubation of Peritan Na *fractions with laccase.* Two fractions of Peritan Na were used: I—which had the highest M_r and IV—which had the lowest M_r. Each fraction at a concn of 1 mg/ml was incubated with the inducible form of *Trametes versicolor* laccase in 10 ml 0.1 M citrate–Pi buffer (pH 4.5) at 20°. After an adequate time of incubation, the reaction was stopped by acidifying the mixture to pH 1.5 with 6 M HCl. Controls containing the boiled enzyme (30 min) were prepared similarly.

Analysis of products. The reaction mixture (5 ml) was applied onto a Sephadex G-50 'fine' column, eluted and detected as described above. New fractions were collected and the M_r of each was determined.

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