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The Effects of Wood Rotting Fungi and Laccase on Destaining of Dyes and KP Bleaching Effluent[*1]

Nam-Seok Cho[*2], J.M. Park[*2], T.H.Choi[*2], A. Matuszewska[*3], M. Jaszek[*3], K. Grzywnowicz[*3], E. Malarczyk[*3], K. Trojanowski[*3] and A. Leonowicz[*3]

ABSTRACT

The ability of several wood rotting fungi for decolorization of two anthracene derivatives, Carminic acid (CA) and Remazol brilliant blue R (RBBR), and hardwood KP bleaching liquor (BL) as well as laccase activities in these fungi were studied. The enzyme activity appeared exclusively in fungi destaining RBBR and CA, but in the case of BL, such relationship was not observed. The laccase enzyme was released into the decolorization media and its inducible (but not constitutive) forms shown destaining activity. The purified inducible forms of *Kuehneromyces mutabilis* and *Pleurotus ostreatus* laccase destained CA. Thus the possible differentiation between specificity of particular LAC forms was confirmed. In addition the nitrogen starvation induced both laccase and CA destaining activities, but the increase was higher for decolorization of CA than LAC activity. Probably LAC would be only partly responsible for decolorization of this dye. This results suggested that purified LACs decolorize CA, however its destaining activities were considerably lower than the activities on syringaldazine.

Key words : carminic acid; Remazol brilliant blue R; bleaching liquor; wood-rotting fungi; laccase; *Kuehneromyces mutabilis*; *Pleurotus ostreatus*

INTRODUCTION

Although lignin is one of most abundant polymers in nature, the mechanism of its biodegradation is only poorly understood. Its study has been slowed down due to the problem of measuring the rate of degradation. A variety of radioactive and unlabelled lignin model compounds have been used to measure ligninolytic activity. Assays, however, are slow. In addition the substrates are usually not commercially available. Therefore, as an alternative method, aromatic dyes are paid attention. It have

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*2 School of Forest Resources, Chungbuk National University, Cheongju 361-763, Republic of Korea
*3 Department of Biochemistry, Maria Curie-Sklodowska University Place 3, PL 20031, Lublin, Poland

been shown that several polymeric dyes were decolorized by *Phanerochaete chrysosporium* under those conditions which favour lignin degradation, suggesting that the ligninolytic activity was responsible for the decolorization of these dyes (Glen & Gold, 1983; Spadaro *et al.* 1992). Besides the most studied *P. chrysosporium*, also *Pleurotus ostreatus* (Platt *et al.*, 1985; Vyas & Molitoris, 1995, Kim *et al.*, 1996; Shin *et al.*, 1997), *Coriolus versicolor* (Livernoche *et al.*, 1983), *Streptomyces sp.* (Pasti & Crawford, 1991) and *Geotrichum candidum* (Kim *et al.*, 1995) are among organisms that have been investigated for decolorizing activity.

Ligninolytic activity of *P. chrysosporium* until recently was closely correlated with lignin peroxidase, but presently attention received also to laccase (LAC, EC 1.10.3.2), since its activity had been found in this mostly investigated fungi (Srinivasan *et al.*, 1995). Consequently it has been proposed that two classes of extracellular enzymes, peroxidases, lignin peroxidase(LiP) and manganese dependent peroxidases(MnP) and LACs, participate in the mechanism of lignin degradation through their ability to catalyze the cleavage of C-C or C-O bonds in lignin or lignin model compounds (Kirk & Hammel, 1992, Hammel *et al.*, 1993, Marzullo *et al.*, 1995). Ollikka *et al.*(1993) demonstrated decolorization of Remazol brilliant blue R (RBBR) dye by LiP of *P. chrysosporium*, and the same LAC activity was shown from *Pycnoporus cinnabarinus* (Schliephake & Lonergan, 1996) and *Cerrena unicolor* (Bekker *et al.*, 1990). The purpose of this study was to screen for wood rotting fungi having destaining ability using black liquor(BL), Carminic acid (CA) and RBBR. The LAC of these fungi and destaining activity of the purified enzyme were also investigated.

MATERIALS AND METHODS

Chemicals

Bovine liver catalase, Brilliant Remazol brilliant blue R (RBBR; 2-anthracenesulfonic acid, 1-amino-9,10-dihydro-9,10-dioxo-4-[[3-[[2-(sulfoxy)-ethenyl] sulfonyl]phenyl] amino]-disodium salt, EP grade) and syringaldazine (4-hydroxy-3,5-dimethoxy benzaldehyde azine, EP grade) were obtained from Aldrich-Chemie (Germany), carminic acid (CA; β-D-glucopyranoside uronic acid, 5,6-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4-oxo-4H-1-benzopyran-7-yl, EP grade) was from TCI (Japan), ferulic acid from Fluka A.G. (Buchs, Switzerland), Sephadex G25 and DEAE-Sephadex A-50 from Pharmacia (Sweden), agar from Junsei Chemical Co. (Japan), and malt and yeast extracts were from DIFCO (USA).

Hardwood BL was prepared from unbleached kraft pulp produced by Donghae Pulp Manufacturing Co. Ltd. Onsan, Korea. The Kappa Number of pulp was 20.0. The mixture was charged (1 hr at room temperature) by 7% chlorine in water (chlorine to pulp ratio, 0.20). Finally a mixture of 4% chlorine in pulp was obtained. For further preparation alkali extraction was made as follows: 180ml water and 0.5g NaOH were added to 200ml flask containing 20g dry (chlorinated) pulp, kept 1 h at 7°C and filtered through the filter paper. The resulted BL was used as a sample for destaining measurement.

Fungi

The cultures of *Abortiporus biennis* (Bull.ex Fr.) Sing. (MC T 060, FCL 123), *C. unicolor* (Bull.ex Fr.) Murr. (MC T 143, FCL 139) and *Gloeophyllum odoratum* (=Wulf.ex Fr.) Imaz. (MC T 143, FCL 124) were obtained from Molitoris Collection, Institute of Botany, University of Regensburg, Germany. *Inonotus obliquus* (Pers.ex Fr.) Pil. (FPD 5042, FCL 19) and *Trametes versicolor* (L.ex Fr.) Pil. (FPD 4838, FCL 20) were from Department of Forest Pathology, Academy of Agriculture, Cracow, Poland. *Kuehneromyces mutabilis* (Schaeff.ex Fr.) Murril. (ACTT 44307, FCL 67), *Phlebia radiata* Fr. (ACTT 64658, FCL 99) and *P. ostreatus* (Jacq.ex Fr.) Kumm. (ACTT 44309, FCL 13) were received from American Type Culture Collection, Rockville, Md., USA. *Lentinus lepideus* Fr. (AMD 64, FCL

39) was from Department of Agricultural Microbiology, Academy of Agriculture, Cracow, Poland. *Pleurotus sajor-caju* (FCTUA 104, FCL 237) was from Laboratory of Forest Product Chemistry, Tokyo University of Agriculture, Tokyo, Japan. *Serpula lacrimans* (Wulf.ex Fr.) Schreet. (BIAU 36, FCL 5) came from Institute of Botany, Ernst-Moritz-Arndt University, Greifswald, Germany.

The fungal cultures were maintained on 2% (wt/vol) malt agar slants in the Fungal Culture Collection of the Department of Biochemistry, University of Lublin, Poland, registrated as FCL in Information Center for European Culture Collections, Braunschweig, Germany. Some of these fungi were also maintained in the School of Forest Resources, Chungbuk National University, Cheongju, Republic of Korea.

Culture conditions and decolorization experiments

Sucrose Malt Yeast(SMY)-agar medium contained 10g sucrose, 10g malt extract, 4g yeast extract and 20g agar in 1000ml deionized water. Fungal strains were inoculated onto 20ml of SMY-agar medium containing 0.05% RBBR, 10% (v/v) BL, 0.05% CA in 20mm-diameter Petri dishes and cultivated at 27℃. Then the decolorization of dyes was evaluated. Fungal growth and decolorizing activities were measured as colony diameter and diameter of decolorized zone in mm.

Determination of laccase and destaining activities

For determination of LAC the shallow stationary cultures, after inoculation with small pieces of mycelium taken from the malt agar slants were incubated at 27℃ in 250ml conical flasks containing 50ml SMY medium coinciding to plate experiments. Afterwards, culture fluids were separated from the mycelia by filtration. LAC activity was measured with syringaldazine as a substrate (molar absorption coefficient = 65,000 $M^{-1}cm^{-1}$) (Leonowicz & Grzywnowicz, 1981) at pH optimum for particular fungi, but with morpholineethanesulfonic acid buffer replaced by 0.1 M citrate phosphate buffer as described Bollag and Leonowicz (1984). To exclude endogenous peroxide, 10 min. preincubation (stirring) of the enzyme sample with catalase (10mg/ml) was performed. The LAC activity was calculated in nkat/l. Destaining (decolorization) activity was assayed in a standard reaction mixture containing 50μl of 1mM CA according to Ulmer *et al.* (1984), 250μl of culture fluid or LAC preparation in distilled water, and 200μl of 0.2 M Na-succinate buffer (pH 6.0). The decrease in absorbency at 456nm was assayed and destaining activity was calculated in nkat/l (molar absorption coefficient = 919 $M^{-1}cm^{-1}$) (Eguchi *et al.*, 1994).

Nitrogen starvation of fungal cultures

For the nitrogen starvation of the fungal cultures, the method of Staszczak *et al.* (1996) based on Lindeberg & Holm (1952) mineral medium was used, but the mycelia were transferred to media deprived L-asparagine after 10 day incubation period. Enzymatic activities were assayed 24 h after exchange of the media.

Isolation and purification of laccase inducible forms

The fermenter cultures of *K. (Pholiota) mutabilis* and *P. ostreatus* based on Lindeberg & Holm (1952) medium were run at 28℃ (Rogalski *et al.*, 1990). To stimulate the production of inducible forms of LAC, ferulic acid was added as an inducer to the concentration of 0.2 mM (Leonowicz *et al.*, 1978). Isolation and purification of inducible LAC forms by chromatography on Sephadex G50 and DEAE-Sephadex A50 columns were done (Leonowicz *et al.*, 1978). The fermenter culture filtrate from the peak of extracellular LAC activity served as a source of the enzyme. Purification factors of 46 and 77 have been obtained for *K. mutabilis* and *P. ostreatus* LAC inducible forms, respectively, after the ion exchange chromatography on DEAE-Sephadex A50 column.

RESULTS AND DISCUSSION

Destaining activities

The results of destaining activities of fungi tested and LAC production by these fungi were shown in Table 1. The majority of our fungi were shown the ability to remove color from BL. Such decolorization treatment of different type of kraft pulp effluent was also observed earlier in the case of *P. chrysosporium* and *T. versicolor* (Archibald *et al.*, 1990; Bajpai & Bajpai, 1993; Bergbauer *et al.*, 1991; Livernoche *et al.*, 1981; Mechna *et al.*, 1995; Terron *et al.*, 1992). Aromatic dyes and phenols which are present in culture media might be caused in cultures inducible conditions. Although our results enlarged the number of destaining strains, the removal of strong dark color from the effluents has not been solved yet, and mechanism and function of the enzyme are still scarcely known. Some reports published decolorization of effluents with fungal LAC. For example, Manzanares *et al.*, (1995) found that production of LAC by *T. versicolor* was correlated to color elimination from kraft pulp effluent.

In this experiment, the destaining activity not only with BL but also with CA and RBBR (Table 1) were measured. Such activity on CA and RBBR was shown interestingly only at LAC producing fungi. In the case of BL, such relationship did not appear, in particular, LAC lacking *L. lepideus* considerably destained this dye (Table 1). Also LiP did not show any decolorization activity on BL (Archibald, 1992). Therefore, the question of which enzyme or enzymes are involved in biological decolorization of pulp effluents is not clear.

Effect of purified laccase

As LAC was found in all fungi destaining CA and RBBR, further experiments were done to clarify the relationship between LAC and decolorization activity. The most strong destaining fungi, *K. mutabilis* and *P. ostreatus,* were selected for the above experiment. Since nitrogen starvation of fungal cultures induces ligninolytic activity (Glenn & Gold, 1983), the manner of nitrogen starvation proposed by Staszczak *et al.* (1996) was applied. According to these authors, transformation of mycelium from nitrogen-rich to nitrogen-deprived medium induces the secondary metabolism of fungi.

Table 2 showed that nitrogen starvation induced both LAC and CA destaining activity, but the increase was higher for decolorization of CA than

Table 1. Destaining activity of fungi on BL, CA and RBBR during 10 day-long plate test in comparison with laccase production by these fungi

Fungus	BL		CA		RBBR		Laccase activity nkat/l
	Growth mm	Decol. mm	Growth mm	Decol. mm	Growth mm	Decol. mm	
A. biennis	18.0	12.5	15.0	15.0	15.0	12.8	1,181
C. unicolor	5.8	3.0	10.0	10.0	10.0	9.0	1,307
G. odoratum	17.0	17.0	18.0	15.0	15.0	11.2	1,169
I. obliquus	14.5	0.0	18.3	0.0	14.2	0.0	0
K. mutabilis	12.4	11.3	13.2	10.3	12.2	9.0	1,340
L. lepideus	12.7	10.2	15.2	0.0	14.1	0.0	0
P. radiata	9.0	9.0	11.3	9.0	10.0	10.0	1,184
P. ostreatus	12.1	10.0	11.3	9.0	11.2	9.0	1,710
P. sajor-caju	12.8	7.9	10.0	10.0	10.0	10.0	1,070
S. lacrimans	13.2	0.0	12.4	0.0	13.4	0.0	0
T. versicolor	15.1	15.1	15.0	11.2	12.8	9.0	1,206

Table 2. Laccase activities and CA destaining activities in nitrogen-rich and nitrogen-deprived cultures of *K. mutabilis* and *P. ostreatus*

Fungus	Laccase (I) nkat/l	CA (II) nkat/l	Laccase (III) nkat/l	CA (IV) nkat/l
K. mutabilis	1,974	97	2,164	174
P. ostreatus	1,826	79	2,743	304

I. Laccase activities after 10 days growth in Lindeberg medium.
II. CA destaining activities after 10 days growth in Lindeberg medium.
III. Laccase activities 24 h after exchange the media into nitrogen-deprived media.
IV. CA destaining activities 24 h after exchange the media into nitrogen-deprived media.

LAC activity. Probably LAC would be only partly responsible for decolorization of this dye. The results presented in Table 3 showed that purified LACs decolorize CA, however its destaining activities were considerably lower than the activities on syringaldazine. When increase of LAC activities on syringaldazine were applied around 7.2 times in the case of *K. mutabilis* and 7.4 times for *P. ostreatus*, the CA decolorizations were increased much slower around 2.8 and 2.9 times for assayed fungi, respectively.

The results presented in Table 2 and Table 3 indirectly pointed that LAC was probably not the only agent causing destaining, although LAC showed destaining activity on CA. As supplements of specific CA destaining enzyme similar to the LAC, the other type enzyme discovered recently by Vyas and Molitoris (1995). This hydrogen peroxide dependent CA destaining enzyme was found in the solid-state culture of *P. ostreatus* in wheat straw. It seems that two destaining enzymes: specific and non-specific (LAC) function in *P. ostreatus* (also in *K. mutabilis* and other fungi) function. The destaining activity of LAC on RBBR was reported earlier in the case of *P. cinnabarinus* (Schliephake & Lonergan, 1996), and *C. unicolor* (Bekker *et al.*, 1990). Our results presented here showed that CA can be destained by LAC. Such unusual activity of fungal LAC is not strange, as the enzyme shows very broad specificity for many hydrogen donors (Bollag & Leonowicz, 1994), including lignin (Leonowicz *et al.*, 1985). The enzyme not only

Table 3. Destaining activity of purified laccase inducible forms with CA in comparison with activity using syringaldazine as substrate

Laccase destaining activity using CA (nkat/l)		Laccase activity on syringaldazine (nkat/l)	
K. mutabilis	*P. ostreatus*	*K. mutabilis*	*P. ostreatus*
23	19	698	540
44	39	2830	2495
65	56	5016	4020
0*	0*	2830*	2743*

*The enzyme preparations were boiled after measuring the activity with syringaldazine.

oxidizes phenolic and methoxyphenolic acids, but also decarboxylates them (Agematu *et al.*, 1993) and modifies their methoxyl groups by demethylation (Leonowicz *et al.*, 1994) or demethoxylation (Potthast *et al.*, 1995). Besides, LAC dechlorinates various chlorophenols (Konishi & Inoue, 1972; Roy-Arcand & Archbald, 1991) and copolymerizes some chloroamines (Simmons *et al.*, 1989) and aminoacids (Liu *et al.*, 1985) with phenolic compounds. LAC probably serves there as a secondary, much less specific agent. In destaining processes, LAC performs the analogical role. Inducible forms (Bollag & Leonowicz, 1984; Leonowicz *et al.*, 1978) of LAC are more specific for various hydrogen donors than constitutive ones. Such phenomenon of differentiated activity between inducible and constitutive forms of LAC was

shown also earlier. Morohoshi *et al.* (1987) and Morohoshi & Haraguchi (1987) found that only one of three LAC forms isolated from *C. versicolor* (synonym of *T. versicolor*) depolymerized certain lignin monomer, while the other two forms of enzyme gave mainly polymerized products.

CONCLUSION

Black liquor which appears during the bleaching or cooking process and has strong dark colour is difficulty to destaining until now in pulp and paper industry. On the other hand, CA and RBBR which are used in industry as the substrates for the production of polymeric dyes, as anthracene derivatives, are very toxic and make troubles with neutralization. Among the systems which may be employed to eliminate the coloured wastewaters, white-rot fungi treatment seems to be promising, because of their capacity to metabolize aromatic by-products. The listed fungal genera tested in this study for decolorization, bring the possibility for the biological application to pulp and paper industry, instead of chemical methods. LAC also seems to be a applicable element.

In this study, the enzyme was released into the decolorization media, and its inducible (but not constitutive) forms were shown destaining activity. Thus the possible differentiation between specificity of particular LAC forms was confirmed. In addition the nitrogen starvation induced both LAC and CA destaining activities, but the increase was higher for decolorization of CA than LAC activity. Probably LAC would be only partly responsible for decolorization of this dye. When increase of LAC activities on syringaldazine were applied around 7.2 times in the case of *K. mutabilis* and 7.4 times for *P. ostreatusu*, the CA decolorizations were increased much slower around 2.8 and 2.9 times for assayed fungi, respectively.

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