Mokchae Konghak 28(4) : 29~40, 2000

Demethoxylation of Milled Wood Lignin and Lignin Related Compounds by Laccase from White-rot Fungus, *Cerrena unicolor*[*1]

A. Leonowicz[*2], J. Rogalski[*2], E. Malarczyk[*2], K. Grzywnowicz[*2], G. Ginalska[*2], J. Lobarzewski[*2], S. Ohga[*3], N. Pashenova[*4], S.S. Lee[*5], and Nam-Seok, Cho[*6]

ABSTRACT

Highly purified *Cerrena unicolor* laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) caused the demethoxylation of milled wood lignin and several lignin related substances. The constitutive form of the enzyme produced extracellularly by *C. unicolor* fermenter culture was isolated and purified by ion-exchange chromatography on the DEAE-Toyopearl column and by affinity chromatography on a ConA-Sepharose and Syringyl-AH-Sepharose 4B columns.

The enzyme was further immobilized on functionalized porous glass (CPG) and keratin coated CPG. The demethylating activity was monitored both by estimation of released methanol and by detection of the level of methoxyl groups (also in some water miscible solvents) after incubation of lignin materials with laccase preparations (free and immobilized). The effects of the incubation time and temperature on the demethoxylating activity of immobilized laccase preparations were also studied.

Keywords : Laccase, constitutive form, *Cerrena unicolor*, demethoxylation, milled wood lignin, lignosulfonates, white-rot fungus

1. INTRODUCTION

White-rot fungi growing on wood or lignin preparations produce several monomeric aromatic compounds, among which, ferulic, syringic and vanillic acids, are predominant (Ishikawa *et al.*, 1963). Also, when wood is degraded by these fungi a significant decrease in the methoxyl content in the remaining lignin can be observed (Trojanowski and Leonowicz, 1963; Leonowicz, 1965; Trojanowski *et al.*, 1966; Ander and Eriksson, 1978; Crawford, 1981; Oki *et al.*, 1982; Tai *et al.*, 1982). This process has been shown to occur with soft-rot (Seifert, 1966), brown-rot (Kirk, 1975) and white-rot fungi (Kirk and Chang, 1974). The white-rotted lignin from

*1 Received on October 4, 2000, accepted on December 11, 2000

*2 Department of Biochemistry, Maria Curie-Sklodowska University, Lublin, Poland
*3 Department of Forest Resources Sciences, College of Agriculture, Kyushu University, Fukuoka, Japan
*4 Institute of Forest, Russian Academy of Science, 660036, Krasnoyarsk, Russia
*5 Department of Biological Education, Korea National University of Education, Chungbuk 360-791, Korea
*6 School of Forest Resources, Chungbuk National University, Cheongju 361-763, Korea

spruce was shown 25% reduction in methoxyl content per C9-unit compared to sound wood (Kirk and Chang, 1974). Also the formation of methanol in fungal environment, pointing on demethoxylating activity, has been observed during growth of white rot fungus, *Phanerochaete chrysosporium* in the media containing synthetic and natural lignins (Ander and Eriksson, 1985) or methoxyphenolic lignin derivatives (Ander *et al.*, 1985).

It has been demonstrated that demethoxylation of lignin and methoxyphenolic acids is involved fungal laccase excreted from white-rot fungus, *Pholiota mutabilis* (Leonowicz, 1965; Leonowicz and Trojanowski, 1965; Trojanowski *et al.*, 1966; Trojanowski and Leonowicz, 1969; Leonowicz *et al.*, 1979). We also found that laccase activity extremely depends on pH, and that demethoxylation processes better go at lower pH (Leonowicz *et al.*, 1984). These results were confirmed with using laccase isolated from several white-rot fungi by Harkin and Obst (1974), Ishihara and Miyazaki (1974), Ander *et al.* (1983), Ishihara (1983) and Lundquist and Kristersson (1985).

As non-induced cultures produce usually very low amount of laccase, most of cited reports were based on laccases isolated from cultures stimulated by several enzyme inducers. For this reason various substances were added to the fungal media, *e.g.* 2,5-xylidine (Ander *et al.*, 1983; Leonowicz *et al.*, 1984), vanillic acid (Trojanowski *et al.*, 1966), ferulic acid (Leonowicz *et al.*, 1979) or syringic acid (Leonowicz *et al.*, 1979), or even maple wood meal (Ishihara and Miyazaki, 1970) and milled wood lignin (MWL) (Leonowicz and Trojanowski, 1965). However, according to our experiences, isolation and purification of laccase from induced cultures cause several problems, such as contamination of the enzyme and laboratory equipment by coloured products of aromatic inducers, and their toxicity for human health, etc.

Recently we performed screening of white-rot fungi for production and inducibility of extracellular laccase and selected the fungus, *Cerrena unicolor* producing constitutive laccase in yield comparable to inductive one excrected by *Trametes versicolor* known as the best laccase producer (Leonowicz *et al.*, 1997). As the constitutive forms of laccases were probably never used for demethoxylation processes, presently, working on the separated and purified constitutive form of laccase from *C. unicolor*, we have unequivocally demonstrated that this enzyme demethoxylates as well several low molecular derivatives of lignin as some lignin preparations.

2. MATERIALS and METHODS

2.1 Strain

The white-rot basidiomycete fungus, *Cerrena unicolor* (Bull. ex Fr.) Murr., Einfarbige Tramete Strain T 143 (Molitoris Collection) was kindly supplied by Dr. H.P. Molitoris (Botanic Institute, University of Regensburg, Germany) and maintained as No. 139 in our Fungal Culture Collection Lublin (FCCL) in Poland and as No. 239 in Fungal Collection Cheongju (FCC) in Korea, on 2% (wt/vol) malt agar slants. MWL was prepared according to the method of Bjorkman (1954 and 1957) with the modification of Trojanowski and Leonowicz (1963). The high molecular fraction of Peritan Na (MW 74,000) which constitutes an adequate model of native lignin was prepared according to Leonowicz *et al.* (1985).

2.2 Culture conditions

The non-induced *C. unicolor* culture, used for

purification of laccase, was grown in a liquid medium. Pieces of *C. unicolor* mycelium (*ca.* 0.5 cm) were transferred from the agar slant into the sterilized liquid medium. Before sterilization, the pH of medium was adjusted by 1 M hydrochloric acid to value 5.6. The culture was grown at 26℃ under static conditions. When the mycelium occupied the whole surface of the liquid mycelial mats were broken by shaking with the beads and the homogenate was transferred into sterilized medium with the same composition. The submerged aerated culture was grown for 12 days at 26°C and antifoam B emulsion was applied, if needed.

2.3 Determination of laccase activity

Laccase activity of the culture fluid and purified free and immobilized enzyme preparations was measured at 20°C according to Bollag and Leonowicz (1984) with syringaldazine as a substrate (Leonowicz and Grzywnowicz, 1981). To exclude endogenous peroxide, the 10 min. preincubation (stirring) of the enzyme sample with catalase (10 mg/ml) was performed. The laccase activities (free and immobilized) were measured using the reaction mixtures containing the enzyme preparation in 0.1 M citrate-phosphate buffer (McIlvaine, 1921), pH 5.5 for *C. unicolor* free and pH 5.7 for immobilized one.

2.4 Determination of protein

The protein content both in the culture fluids and in laccase preparations was determined according to Ehresmann *et al.* (1973). Bovine albumin was used as a standard. The quantity of protein bound on to the supports was calculated by subtracting the protein in the combined original filtrate and wash liquid of the immobilized enzyme from the protein used for immobilization.

2.5 Laccase preparation

For the constitutive laccase purification, the method of Leonowicz *et al.* (1997) with some our and Gianfreda *et al.* (1998) modifications was applied as follows. The aerated cultures at the tops of laccase activity were filtered through Miracloth (Calbiochem, Lucerne, Switzerland). Each filtrate was desalted on the Sephadex G-25 column. The enzyme solutions were concentrated to *ca.* one tenth of the volume at 4°C with an Amicon ultrafiltration system equipped with a filter type PTGC (pore size 10,000 NMWL) and dialysed 7 times to the deionized water and (before the end of dialysis) to 0.1 M phosphate buffer, pH 6. Then 25 ml portions of enzyme preparations were applied onto the ConA-Sepharose columns (7 × 1.5 cm), which were then washed with 0.1 M phosphate buffer, until all unbound proteins were removed. The specifically bound proteins, including laccase, were eluted from the column with 20% sucrose (or, even better, with 10% α-methyl mannoside) in 0.1 M phosphate buffer, pH 6. The fractions showing the highest laccase activity were concentrated by ultrafiltration, poured onto DEAE-Toyopearl column (25 × 1.5 cm) and eluted by a linear gradient of 0 to 0.35 M NaCl in 5 mM TRIS/HCl buffer, pH 6.0. The fractions around the top of laccase activity, eluted by NaCl were collected, dialysed to 0.5 mM TRIS/HCl buffer, pH 6, and applied onto a column of AH-sepharose 4B coupled to syringaldehyde. The enzyme was eluted by 0.5 M ammonium sulphate dissolved in the same buffer, pH 6.0, and stored as freeze-dried.

2.6 Laccase immobilization

The porous glass beads (CPG), obtained from Cormey-Lublin, Poland, were stirred in concentrated nitric acid, washed thoroughly with dist. water (Sarkar *et al.*, 1989). The support was silanized by 3-aminopropyltriethoxysilane (APTES) (Robinson *et al.*, 1971) and activated by glutaraldehyde (Lappi *et al.*, 1976) or coated with the feathers keratin (possessing active peptide groups to coat inorganic supports). Keratin was joined to CPG by means an "adhesive" method as follows. It was dissolved in dimethylsulphoxide according to Lobarzewski *et al.* (1984), precipitated with frozen acetone and kept in refrigerator at 4°C. After 24 hrs the acetone was removed by centrifugation and the keratin pellet was washed with distilled water several times and mixed with water in weight ratio 3 : 100. The obtained keratin—water mixture was mixed (stirring continuously) with inorganic support in the ratio 1 : 1 (volume per weight), evaporated to dryness and additionally dried in air. Laccase was coupled to silanized and activated by glutaraldehyde CPG according to the method of Leonowicz *et al.* (1988), and the enzyme coupling to keratin coated silanized CPG was performed according to Ginalska *et al.* (1999). The final preparations were filtered, washed with 0.1 M phosphate buffer pH 7, suspended in 0.1 M phosphate buffer, pH 6, and stored at 4°C.

2.7 Determination of demethoxylating and oxidative activities of laccase

Methoxyphenolic acid (20 μM) was added to 4 ml of 1 mM acetate buffer pH 4.0 or 1 mM citrate-phosphate buffer pH 6.0 containing laccase (250 μg of both free and immobilized proteins), the volumes were adjusted to 5 ml with the same buffer respectively, and the samples were incubated for 3 hrs at 30°C. The MWL was dissolved in 5% (w/w) dimethylsufoxide (DMSO) and mixed with 1 mM acetate buffer pH 4.5 or 1 mM McIlvaine buffer pH 6.0 containing laccase (250 μg of protein) to give a final concentration of 1 mg ml^{-1} according to Leonowicz *et al.* (1999). The high molecular fraction of Peritan Na at a final concentration of 1 mg ml^{-1} was mixed with 1 mM acetate buffer pH 4.5 or 1 mM McIlvaine buffer pH 6.0 also containing laccase (250 μg of each protein). After evaporation of the reaction mixtures at 40°C under vacuum to dryness, the degree of demethoxylation was determined by measuring the content of non-dissociated methoxyl groups from the aromatic rings of the substrates; the assay was performed with hydriodic acid according to Brauns (1952) with the modification introduced by Leonowicz (1965). The oxidative activity of laccase was determined polarographically according to Leonowicz *et al.* (1979).

2.8 Effect of temperature and pH on demethoxylation activity of laccase

Demethoxylation activity of free and immobilized laccase was assayed at different temperatures (4 to 60°C), at pH 4.5 and 6.0, in 1 mM acetate and McIlvaine buffers respectively.

2.9 Effect of organic solvents

The demethoxylation activity of laccase (free and immobilized) was studied in the presence of three organic solvents (30% dioxane, ethylene glycol or formamide). The blank samples containing solvents but devoid laccase substrates were analyzed (Luterek *et al.*, 1998).

3. RESULTS and DISCUSSION

The *C. unicolor* extracellular laccase was isolated and purified from 12 day old cultures. The purification procedure was based on ion-exchange chromatography on the DEAE-Toyopearl column and affinity chromatography on the ConA-Sepharose and Syringyl-AH-Sepharose 4B (Table 1). Using this method the enzyme was purified more than 100-fold (Table 1). It seems that our laccase preparation, isolated from *C. unicolor*, and using 5 steps of purification, was sufficiently purified to carry out the followed experiments. The purified laccase was immobilized on the silanized porous glass beads and keratin coated CPG, after activation with glutaraldehyde. As a result of the bonding procedure, 81.2% of protein and 82.3% of laccase activity and 61.3% of protein and 95.3% of laccase activity were coupled to the CPG and keratin coated CPG supports, respectively. The final preparations contained *ca.* 2.2 and 1.6 mg protein per 1 gram of glass supports. Their specific activities were 62.3 and 73.4 units/mg protein, respectively. These results are in agreement with those reported by Ginalska *et al.* (1999).

Many reports indicated that laccase can demethoxylate lignin and its derivatives (Leonowicz, 1965; Leonowicz and Trojanowski, 1965; Trojanowski *et al.*, 1966; Trojanowski and Leonowicz, 1969; Leonowicz *et al.*, 1979; Harkin and Obst, 1974; Ishihara and Miyazaki, 1974; Ander *et al.* 1983; Leonowicz *et al.*, 1984 ; Lundquist and Kristersson, 1985). Besides, we found that at different pH values various products are formed, and that demethoxylation processes better go at lower pH values (Leonowicz *et al.*, 1984). The results presented in Tables 2, 3 and 4 unequivocally confirm the results regarded demethoxylation activity of laccase and show that in all cases of lignin-related substances the process much better progressed at pH 4.5 than at pH 6.0. This fact means that demethylation occurred mainly at pH around 4.5, whereas at pH 6.0 the release of methoxyl groups was significantly reduced. However, at the some experiments, the oxidative activities of laccase were not very differentiated. Probably their pH optima occur between pH 4.5 and pH 6.0 (Luterek *et al.*, 1998), whereas pH optima for demethoxylation activities are much closer to pH 4.5 than to pH 6.0. We also confirmed our earlier result regarding the ratio between the oxidative and demethoxylation activity of fungal laccase (Leonowicz *et al.*, 1979).

The results in Tables 2, 3 and 4 show that the oxidative/demethoxylation ratio of *C. unicolor* laccase activity was very similar to the result from *Pholiota mutabilis* laccase (Leonowicz *et al.*, 1979). It is of considerable interest that the much amounts of methanol released on demethoxylation of several lignin related substances correspond to the decrease of methoxyl groups in the reaction media. This confirms our

Table 1. Isolation and purification of *C. unicolor* constitutive laccase.

Purification step	Activity		Yield (%)	Purification (fold)
	Total (nkat/vol)	Specific (total/mg prot)		
Filtrate	67245	47	100.0	1.0
Sephadex G-25	39478	86	58.7	1.8
Ultrafiltration	35340	174	52.5	3.7
ConA-Sepharose	30175	1911	47.2	47.0
DEAE-Toyopearl	8792	3121	13.1	66.4
Syringyl-AH-Sepharose 4B 67245	3984	5012	6.7	106.6

earlier results obtained with using of *P. mutabilis* laccase (Leonowicz *et al.*, 1979).

Bae *et al.* (1995) and Bae and Kim (1996) examined the activity of laccase (and Mn-peroxidase) on commercial lignosulfonates at various conditions of pH, and reported that degradation occurred primarily at pH 4.5, whereas polymerization was dominant at pH 6.0. Ishihara and Miyazaki (1976), Ishihara (1983) and Leonowicz *et al.* (1984) reported that demethylation of syringic and vanillic acids was pH-dependent and, in the case of demethoxylation processes, its lower value was much more significant than higher one. During demethoxylation, laccase acts on aryl-*O*-alkyl bonds. If the enzyme is able to attack this 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 (Leonowicz *et al.*, 1999a). It was also suggested (Ishihara, 1980; Leonowicz *et al.*, 1985) that polymerization and depolymerization activities of laccase can result from the oxidative ability of the enzyme toward phenols or polyphenols, and radicals formed react with each other causing both processes. Such phenolic substrates could be produced in the course of demethoxylation as the first step of lignin degradation (Leonowicz *et al.*, 1985). On the other hand it was shown that the pH of culture medium is critical for lignin degradation, and its optimum is at around pH 4.5 (Kirk *et al.*, 1978).

When laccase works at various pH values,

Table 2. Oxidative and demethoxyylating activity of the constitutive laccase isolated from *C. unicolor*.

Lignin related substances	Activity (nmol/min/mg laccase protein)						The oxidative /demethoxylating activity ratio
	Oxidative		Demethoxylating				
			Decrease of $-OCH_3$		Increase of CH_3OH		
	pH 4.5	pH 6.0	pH 4.5	pH 6.0	pH 4.5	pH 6.0	
Acetovanillone	1145	1139	74	8	72	6	15.4
Acetoveratrone-α-vanillic acid ether	197	116	46	6	39	4	4.3
Acetoveratrone-α-ferulic acid ether	176	189	39	5	42	5	4.5
Milled wood lignin	95	49	12	3	10	4	7.9
Caffeic acid	1190	1210	0	0	0	0	-
Coniferyl alcohol	1178	1228	91	8	88	6	12.9
Ferulic acid	1223	1417	93	9	98	8	13.1
Gallic acid	1167	1137	0	0	0	0	-
Peritan Na (high molecular fraction)	67	73	16	4	13	4	4.2
Sinapic acid	1154	1167	65	6	58	5	17.7
Syringaldehyde	1167	1183	89	9	79	8	13.1
Syringic acid	1176	1179	94	8	89	8	12.5
Vanillic acid	1193	1188	116	11	109	10	10.3
Vanillin	1197	1119	196	13	188	11	6.1
Vanillyl alcohol	1194	1189	107	8	99	9	11.2
Veratric acid	0	0	157	12	165	13	0.070
Veratraldehyde	0	0	97	8	91	6	0.092
Veratryl alcohol	0	0	137	9	141	8	0.051

Table 3. Oxidative and demethoxylating activity of the constitutive laccase isolated from *C. unicolor* and immobilized on CPG.

Lignin related substances	Activity (nmol/min/mg laccase protein)						The oxidative /demethoxylating activity ratio
	Oxidative		Demethoxylating				
			Decrease of $-OCH_3$		Increase of CH_3OH		
	pH 4.0	pH 6.0	pH 4.0	pH 6.0	pH 4.0	pH 6.0	
Acetovanillone	1324	1243	77	5	69	4	17.2
Acetoveratrone-α-vanillic acid ether	231	142	44	6	41	5	5.3
Acetoveratrone-α-ferulic acid ether	198	198	32	4	39	7	6.2
Milled wood lignin	110	43	10	4	12	5	11.0
Caffeic acid	1256	1245	0	0	0	0	-
Coniferyl alcohol	1265	1296	94	6	97	7	13.5
Ferulic acid	1299	1359	97	7	101	9	13.4
Gallic acid	1247	1243	0	0	0	0	-
Peritan Na (high molecular fraction)	74	77	15	3	16	7	4.9
Sinapic acid	1234	1198	68	5	54	3	18.1
Syringaldehyde	1241	1286	81	7	76	5	15.3
Syringic acid	1221	1272	98	7	87	4	12.5
Vanillic acid	1272	1247	128	10	101	11	9.9
Vanillin	1251	1145	124	12	194	13	10.1
Vanillyl alcohol	1231	1176	122	7	103	7	10.1
Veratric acid	0	0	117	15	169	11	0.076
Veratraldehyde	0	0	94	6	88	5	0.120
Veratryl alcohol	0	0	112	8	147	9	0.062

depolymerization processes occur mainly at pH optimum for the enzyme activity. It appears usually between pH 4.0 and 5.0. Then various radicals are easily released from lignin substrates, react each other and also produced very active hydroxyl radicals by laccase (during quinone cycling) (Ishihara and Miyazaki, 1976; Ishihara, 1983; Leonowicz *et al.*, 1985). Such activity results finally in lignin degradation (Leonowicz *et al.*, 1985; Leonowicz *et al.*, 1999b). Polymerization occurs usually at not optimum pH for the enzyme activity, when radicals production is rather low (Ishihara, 1983).

The time and temperature dependent on liberation of methoxyl groups from chosen methoxyphenolic substrates by immobilized laccase is shown in Figure 1 and Figure 2, respectively. For this reason instead of free laccase, more resistant immobilized enzyme for incubation time and temperature was used (Ginalska *et al.*, 1999). The results showed that the release of methoxyl groups primarily decreased with increasing molecular weight of substrates. These results seem to be in agreement with the data reported by Ander *et al.* (1985). The authors studied methanol production resulting from the demethoxylation of lignin-related substances by *Phanerochaete chrysosporium*. They suggested that demethoxylation activity may be responsible for either hydrogen peroxide produced by glucose oxidases or lignin peroxidase, which shows demethoxylation ability (Kersten *et al.*, 1985). Presently, we may suggest that probably also laccase took share in this process. The authors did not consider this enzyme probably because its production by *P. chrysosporium* was unknown at that time (laccase was discovered in this fungus ten years latter by Srinivasan *et al.* in 1995).

Table 4. Oxidative and demethoxylating activity of the laccase isolated from *C. unicolor* and immobilized on keratin coated CPG.

Lignin related substances	Activity (nmol/min/mg laccase protein)						The oxidative demethoxylating activity ratio
	Oxidative		Demethoxylating				
			Decrease of $-OCH_3$		Increase of CH_3OH		
	pH 4.0	pH 6.0	pH 4.0	pH 6.0	pH 4.0	pH 6.0	
Acetovanillone	1314	1375	79	7	74	5	16.6
Acetoveratrone-α-vanillic acid ether	179	156	55	7	54	6	5.1
Acetoveratrone-α-ferulic acid ether	217	214	39	5	42	6	5.6
Milled wood lignin	123	51	13	4	14	6	9.5
Caffeic acid	1327	1274	0	0	0	0	-
Coniferyl alcohol	1298	1317	97	5	101	8	13.4
Ferulic acid	1352	1374	112	8	118	10	12.1
Gallic acid	1326	1311	0	0	0	0	-
Peritan Na (high molecular fraction)	137	98	16	4	18	5	8.6
Sinapic acid	1285	1264	72	6	67	4	17.8
Syringaldehyde	1287	1304	85	8	79	6	15.1
Syringic acid	1356	1298	113	8	99	5	12
Vanillic acid	1397	1389	137	18	129	10	10.2
Vanillin	1274	1198	129	11	134	11	9.9
Vanillyl alcohol	1289	1194	127	9	133	4	10.1
Veratric acid	0	0	131	13	149	11	0.053
Veratraldehyde	0	0	94	6	88	4	0.120
Veratryl alcohol	0	0	116	7	155	8	0.043

Finally we investigated laccase demethoxylation activity in some water miscible organic solvents (Table 5). Previously Milstein *et al.* (1989) have shown that the enzyme may function in such unnatural condition oxidizing aromatic compounds. Moreover, probably polymerization processes of lignin derived phenoxy radicals, released by laccase or peroxidase, might be diminished in some organic solvents in comparison to the aqueous solutions (Dordick *et al.*, 1986; Kazandjian and Klibanov, 1985). In this reason we expected better demethoxylation activity of laccase in these solvents than in water. Our earlier experiments showed that each solvent tested in an anhydrous form caused an inhibitory effect on the activity of laccase (Luterek *et al.*, 1998). Table 5 was shown the effect of three selected organic solvents, at 30% concentration, on the laccase caused releasing of

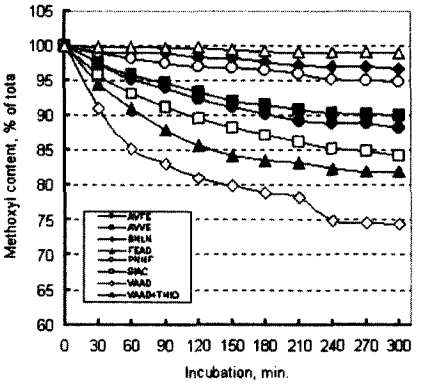

Fig. 1. Liberation of methoxyl groups from lignin and its derivatives during incubation at 20° C with *C. unicolor* laccase immobilized on CPG.

*AVFE, acetoveratrone-α-ferulic acid ether; AVVE, acetoveratrone-α-vanillic acid ether; BNLN, milled wood lignin; FEAD, ferulic acid; PNHF, Peritan Na (high molecular fraction); SIAC, sinapic acid; VAAD, vanillic acid; VAAD+THIO, vanillic acid + thioglycolic acid.

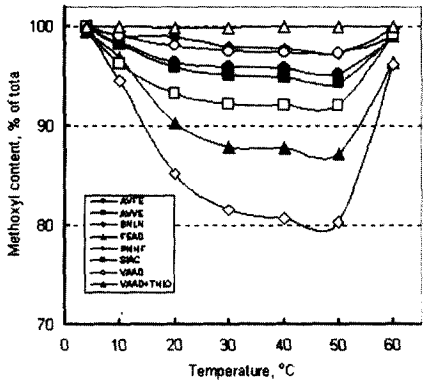

Fig. 2. Liberation of methoxyl groups from lignin and its derivatives during 60 min incubation with *C. unicolor* laccase immobilized on keratin coated CPG.

methoxyl groups from chosen lignin related substances. No meaningful differences can be seen between results obtained in this condition in comparison to one without organic solvents (see Figure 1 and Figure 2). It is not surprising, as earlier we found that these organic solvents in a concentration of 30% almost did not influence laccase regular activity (Luterek *et al.*, 1998).

4. CONCLUSION

Authors performed screening of white-rot fungi for production and inducibility of extracellular laccase and selected the fungus, *Cerrena unicolor* producing constitutive laccase in yield comparable to inductive one excreted by *Trametes versicolor*. As the constitutive forms of laccases were separated, purified and demonstrated for demethoxylation of several low molecular derivatives of lignin as some lignin preparations. The laccase was purified in high yield as a constitutive form, displayed demethoxylation activity similar to those of other fungal laccases. All presented results strongly indicate that the extracellular *C. unicolor* laccase demethoxylates as well milled wood lignin as lignin related dimers and monomers.

ACKNOWLEDGEMENT

This work was mainly supported by Korea Research Foundation Grant (2000-042-G00016), Brain Pool Program (992-4-31) of KOFST (2000), 99Russia-Invitation-04-003 of KISTEP (2000) and partly European Community (RTD5 EC Proposal No. ICA2-1999-10027-Acronym PUBLIER) and KBN (6PO4B 011515 BS/BiNoZ/4, BW/BiNoZ4).

Table 5. Liberation of methoxyl groups from lignin and its derivatives during 60 min incubation at 20°C in organic solvents with *C. unicolor* laccase immobilized on keratin-CPG.

Solvents*2	-OCH_3 (% of total)							
	AVFE*1	AVVE	BNLN	FEAD	PNHF	SIAC	VAAD	VAAD+THIO
Dioxane	96.8	95.9	98.3	90.5	98.3	93.8	85.5	100
Ethylene glycol	96.1	95.6	98.1	90.3	98.1	93.4	85.3	100
Formamide	96.3	95.8	98.9	90.6	98.4	93.7	85.7	100

*1 AVFE, acetoveratrone-alpha-ferulic acid ether; AVVE, acetoveratrone-α-vanillic acid ether; BNLN, milled wood lignin; FEAD, ferulic acid; PNHF, Peritan Na (high molecular fraction); SIAC, sinapic acid; VAAD, vanillic acid; VAAD+THIO, vanillic acid + thioglycolic acid.

*2 All solvents were applied as the 30% mixture in 4.5 mM acetic buffer.

REFERENCES

1. Ander, P. and K.-E. Eriksson. 1985. Methanol formation during lignin degradation by *Phanerochaete chrysosporium*. Appl. Microbiol. Technol. 21, 96-102.
2. Ander, P., E. R. M. Eriksson and K.-E. Eriksson. 1985. Methanol production from lignin-related substances by *Phanerochaete chrysosporium*. Physiol. Plant. 65, 317-321.
3. Ander, P. and K.-E. Eriksson. 1978. Lignin degradation and utilization by microorganisms. In: Progr. Ind. Microbiol, Vol. 14. Ed. M.J. Bull, Elsevier, Amsterdam. pp. 1-58.
4. Ander, P., K.-E. Eriksson and H.-S. Yu. 1983. Vanillic acid metabolism by *Sporotrichum pulverulentum*: evidence for demethoxylation before ring cleavage. Arch. Microbiol. 136, 1-6.
5. Bae, H. J. and Y. S. Kim. 1996. Degradation of ligninsulfonates by simultaneous action of laccase and Mn-peroxidase. In: Biotechnology in the Pulp and Paper Industry. Eds. E. Srebotnik, K. Messner. Facultas-Universitatsverlag, Vienna, pp. 393-396.
6. Bae, H. J., S. K. Yoon and P. M. Sung. 1995. Degradation characteristics of liginsulfonate by laccase and Mn-peroxidase. Mokchae Konghak 23, 27-32.
7. Bjorkman, A. 1954. Isolation of lignin from finely divided wood with neutral solvents. Nature 174, 1057-1058.
8. Bjorkman, A. 1957. Lignin and carbohydrate complexes extraction from wood meal with neutral solvents. Ind. Eng. Chem. 49, 1395-1399.
9. Bollag, J.-M. and A. Leonowicz. 1984. Comparative studies of extracellular fungal laccases. Appl. Env. Microbiol. 48, 849-854.
10. Brauns, F. E. 1952. Methoxyl determination in lignin derivatives. The chemistry of lignin. Academic Press, New York, pp. 744-745.
11. Crawford, R. L. 1981. Lignin biodegradation and transformation. Wiley-Intedscience, New York.
12. Dordick, J. S., M. A. Marletta nd A. M. Klibanov. 1986. Peroxidases depolymerize lignin in organic media but not in water. Proc. Natl. Acad. Sci.. 83, 6255-6257.
13. Ehresmann, B., P. Imbault and J. H. Weil. 1973. Spectrophotometric determination of protein concentration in cell extracts containing tRNAs and rRNAs. Anal. Biochem. 54, 454-463.
14. Gianfreda, L., F. Sannino, M. T. Filazzola and A. Leonowicz. 1998. Catalytic behavior and detoxifying ability of a laccase from the fungal strain *Cerrena unicolor*. J. Mol. Cat. 4, 13-23.
15. Ginalska, G., J. Lobarzewski, Nam-Seok Cho, J. M. Park, M. Jaszek and A. Leonowicz. 1999. Keratin as a matrix for laccase immobilization. In: New horizon of bioscience in forest products fields. Proc. Internat'l Conf. Forest Sci., Chungbuk Nat. Univ., Cheongju, Korea, pp. 164-174.
16. Harkin, J. M. and J. R. Obst. 1974. Demethylation of 2,4,6-trimethoxyphenol by phenol oxidases. A model for chromophore formation in wood and pulp. TAPPI 57, 118-121.
17. Harkin, J. M., M. J. Larsen and J. R. Obst. 1974. Use of syringaldazine for detection of laccase in sporophores of wood rotting fungi. Mycologia (N.Y.) 66, 469-476.
18. Ishihara, T. and M. Miyazaki. 1974. Demethylation of lignin and lignin models by fungal laccase. Mokuzai Gakkaishi 20, 39-41.
19. Ishihara, T. and M. Miyazaki. 1970. Formation a new crystalline compound from vanillic acid by the action of crude enzyme of *Polyporus versicolor*. Mokuzai Gakkaishi 16, 181-184.
20. Ishihara, T. and M. Miyazaki. 1976. Oxidation of syringic acid by fungal laccase. Mokuzai Gakkaishi 22, 371-375.
21. Ishihara, T. 1983. Effect of pH in the oxidation of syringic acid by fungal laccase. Mokuzai Gakkaishi 29, 801-805.
22. Ishihara, T. 1980. The role of laccase in lignin degradation. In: Lignin degradation: microbiology, chemistry and potential applications. Eds. T. K.

Kirk, T. Higuchi and H. M. Chang. CRC Press, Inc., Boca Raton, Fl. pp. 17-31.

23. Ishikawa, H., W. J. Schubert and F. F. Nord. 1963. Investigations on lignins and lignification XXVIII. The degradation by *Polyporus versicolor* and *Fomes fomentarius* of aromatic compounds structurally related to softwood lignin. Arch. Biochem. Biophys. 100, 140-149.
24. Kazandjian, R. Z. and A. M. Klibanov. 1985. Regioselective oxidation of phenols catalyzed by polyphenol oxidase in chloroform. J. Am. Chem. Soc. 107, 5448-5450.
25. Kersten, P. J., M. Tien, B. Kalyananraman and T. K. Kirk. 1985. The ligninase of Phanerochaete chrysosporium generates cation radicals from methoxybenzenes. J. Biol. Chem. 260, 2609-2612.
26. Kirk, T. K. and H.-M. Chang. 1974. Decomposition of lignin by white-rot fungi. I. Isolation of heavily degraded lignin from decayed spruce. Holzforschung 28, 217-222.
27. Kirk, T. K. 1975. Effects of a brown-rot fungus, Lenzites trabea, on lignin in spruce wood. Holzforschung 29, 99-107.
28. Lappi, D. A., F. E. Stolzenbach, N. O. Kaplan and M. D. Kamen. 1976. Immobilization of hydrogenase on glass beads. Biochem. Biophys. Res. Commun. 69, 878-884.
29. Leonowicz, A. and J. Trojanowski. 1965. Exoenzymes in fungi degrading lignin. Acta Microbiol. Polon. 14, 55-61.
30. Leonowicz, A. and K. Grzywnowicz.1981. Quantitative estimation of laccase forms in some white-rot fungi using syringaldazine as a substrate. Enzyme Microb. Technol. 3, 55-58.
31. Leonowicz, A. 1965. Badania nad rozkladaniem ligniny przez grzyb *Pholiota mutabilis*. Doctors Dissertation, pp. 45-47, UMCS, Lublin.
32. Leonowicz, A., G. Szklarz and M. Wojtas-Wasilewska. 1985. The effect of fungal laccase on fractionated lignosulphonates (Peritan Na). Phytochemistry 24, 393-396.
33. Leonowicz, A., J. M. Sarkar and J. M. Bollag. 1988. Improvement in stability of an immobilized fungal laccase. Appl. Microbiol. Biotechnol. 29, 129-135.
34. Leonowicz, A., J. Rogalski, M. Jaszek, J. Luterek, M. Wojtas-Wasilewska, E. Malarczyk, G. Ginalska, M. Fink-Boots and Nam-Seok Cho. 1999a. Cooperation of fungal laccase and glucose-1-oxidase in transformation of Bjorkman lignin and some phenolic compounds. Holzforschung (in press).
35. Leonowicz, A., K. Grzywnowicz and M. Malinowska. 1979. Oxidative and demethylating activity of multiple forms of laccase from *Pholiota mutabilis*. Acta Biochim. Polon. 26, 431-434.
36. Leonowicz, A. L. Gianfreda, J. Rogalski, M. Jaszek, J. Luterek, M. Wojtas-Wasilewska, E. Malarczyk, A. Dawidowicz, M. Fink-Boots, G. Ginalska, M. Staszczak and Nam-Seok Cho. 1997. Appearance of laccase in wood-rotting fungi and its inducibility. Mokchae Konghak 25, 29-36.
37. Leonowicz, A., L. Gianfreda, J. Rogalski, M. Jaszek, J. Luterek, M. Wojtas-Wasilewska, E. Malarczyk, A. Dawidowicz, M. Fink-Boots, G. Ginalska, A. Leonowicz and N. S. Cho. 1997. Purification of extracellular Cerrena unicolor laccase by means of affinity chromatography. J. Korea Tappi 29, 7-17.
38. Leonowicz, A., Nam-Seok Cho, J. Luterek, M. Wojtas-Wasilewska, M. Hofrichter and J. Rogalski 1999b. Is lignin transformed by fungal laccase? In: New horizon of bioscience in forest products fields. Proc. Internat'l Conf. Forest Sci., Chungbuk Nat. Univ., Cheongju, Korea, pp. 51-100.
39. Leonowicz, A., R. U. Edgehill and J.-M. Bollag. 1984. The effect of pH on the transformation of syringic and vanillic acids by the laccase of *Rhizoctonia praticola* and *Trametes versicolor*. Arch. Microbiol. 137, 89-96.
40. Lobarzewski, J., A. Paszczynski, T. Wolski and J. Fiedurek. 1984. Keratin and polyamide-coated inorganic matrices as supports for glucoamylase immobilization. Bioch. Bioph. Res. Comm. 121, 220-228.

41. Lundquist, K. and P. Kristersson. 1985. Exhaustive laccase-catalysed oxidation of lignin model compounds (vanillyl glycol) produces methanol and polymeric quinoid products. Biochem. J. 229, 277-279.
42. Luterek, J., L. Gianfreda, M. Wojtas-Wasilewska, Nam-Seok Cho, J. Rogalski, M. Jaszek, E. Malarczyk, M. Staszczak, M. Fink-Boots and A. Leonowicz. 1998. Activity of free and immobilized extracellular *Cerrena unicolor* laccase in water miscible organic solvents. Holzforschung 52, 589-595.
43. McIlvaine, T. C. 1921. A buffer solution for colorimetric comparison. J. Biol. Chem. 49, 183-186.
44. Milstein, O., B. Nicklas and A. Huttermann. 1989. Oxidation of aromatic compounds in organic solvents with laccase from *Trametes versicolor*. Appl. Microbiol. Biotechnol. 31, 70-74.
45. Oki, T., Y. Senba and H. Ishikawa. 1982. The biodegradation of softwood lignin and guaiacylglycerol-β-guaiacyl ether by extra-cellular enzyme in Shiitake Lentinus edodes (Berk.) Sing. Mokuzai Gakkaishi 28, 800-808.
46. Robinson, O. J., P. Dunnill and M. D. Lilly. 1971. Porous glass as a solid support for immobilization or affinity chromatography of enzymes. Biochim. Biophys. Acta 242, 659-661.
47. Sarkar, J. M., A. Leonowicz and J.-M. Bollag. 1989. Immobilization of enzyme on clays and soils. Soil Biol. Biochem. 21, 223-230.
48. Seifert, K. 1966. Die chemische Veranderung der Buchenholz-Zellwand durch Moderfaule (*Chaetomium globosum* Kunze). Holz Roh. u. Werkstoff 24, 185-189.
49. Srinivasan, C., T. M. D'Suoza, K. Boominathan and C. A. Reddy. 1995. Demonstration of laccase in the white-rot Basidiomycete *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 61, 4274-4277.
50. Tai, D., I. Terazawa, V.-B. Huunh, C.-L. Chen, H.-M. Chang and T. K. Kirk. 1982. Degradation of lignin in birth wood by the white-rot fungus *Phanerochaete chrysosporium*. 1982 TAPPI Res. Div. Conf., TAPPI Proceedings, pp. 263-272.
51. Trojanowski, J., A. Leonowicz and B. Hampel. 1966. Exoenzymes in fungi degrading lignin. II. Demethoxylation of lignin and vanillic acid. Acta Microbiol. Polon. 15, 17-22.
52. Trojanowski, J. and A. Leonowicz. 1963. Investigations on the degradation of lignin by *Pholiota mutabilis*. Ann. Univ. M. Curie-Sklodowska, Lublin (C) 18, 441-452.
53. Trojanowski, J. and A. Leonowicz. 1969. The biodegradation of lignin by fungi. Microbios 3, 247-251.