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Purification of extracellular *Cerrena unicolor* laccase by means of affinity chromatography[*1]

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친화크로마토그래프법을 이용한 Cerrena unicolor laccase의 정제특성[*1]

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요 약

균체가 생산하는 laccase 효소가 다방면으로 이용되면서, 이 효소를 균체로부터 대량으로 생산하고 분리.정제하여야 할 필요성이 대두되고 있다. 아울러 이 효소의 활성을 유도하기 위한 inducer 로서 2,5-xylidine 이 주로 사용되고 있는바, 이 xylidine 의 유독성이 인정되면서 사람에게 독성을 주지 않는 환경친화적 inducer 의 검색이 필요하게 되었다.

본 연구에서는 백색부후균 *Cerrena unicolor* 가 분비하는 laccase 효소의 유도를 위한 inducer 로서 ferulic acid 를 사용하였으며, 균체로부터 생산 및 분리된 laccase 효소의 정제특성을 구명코자 하였다. 본효소(constitutive enzyme)로서 I 및 II 를, ferulic acid 를 inducer 로 사용한 경우 inducing 효소 III 을 분리.정제하였다. 본 효소 I 및 II 의 Michaelis 정수는 각각 737 M, 716 M 이었고, inducing 효소 III 은 167 M 로서, 기질에 대한 높은 친화성을 보여주고 있다. 분자량도 각각 65 kD, 63 kD 였으며, inducing 효소 III 은 59 kD 였다. 두 효소 모두 15 - 19% 의 당 및 단백질분자당 4 M 의 동(Cu)을 함유하고 있었다. 정제효율은 효소 I 및 II 가 10.1%, 9.4% 였으며, III 은 3.2% 였다. 모든 효소의 최적 pH 는 5.5 였으며, 최적온도는 비교적 높은 40℃ 였다.

Key words: white-rot fungus; laccase; *Cerrena unicolor*; purification; affinity chromatography

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1. INTRODUCTION

Fungal laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2). occurs in various classes of fungi[1]. The enzyme catalyzes molecularly the removal of an electron and a proton from a hydroxyl group to form a free phenoxy radical of o- and p-diphenols as well as aromatic amines. Not only oxidizes a large variety of aromatic hydrogen donors, mainly phenolic and methoxyphenolic acids, but also decarboxylates them[1] and modifies their methoxyl groups by demethylation[2] or demethoxylation3). These reactions constitute an important step in the initial transformation of lignin polymers[1-3].

Laccase is involved in several, applicable also in biotechnology, physiological processes. For example, it is engaged in the development of fungal fruit bodies[4], pigmentation of fungal spores[5], pathogeneity[6], sexual differentiation[7] or rhizomorph formation[8]. Laccase can be used as a free enzyme and an immobilized preparation both in water and in some organic solvents, improving in several biotechnological processes this way[9,10].

Of possible applications, the enzyme is considered, for example, to be a bleaching agent in the pulp and paper technology[11], a stabilizer during the must and wine processing[12] or as a dechlorinating factor[13]. Its broad activity on hydrogen donors provides opportunities for detoxification of some terrestrial pollutants[14] as well as for removing certain phenolic and other aromatic compounds from natural and industrial wastewaters[15]. The oxidation of phenolics generates phenoxy radicals and quinoid intermediates which are subsequently transformed to dimers and insoluble polymers with aromatic amines (e.g. chloroamines)[16] or aminoacids[17]. After sedimentation such polymers may be removed from the water environment. Terrestrial pollutants have a chance to be oxidized by laccase immobilized in the soil fractions to less toxic polymers[18]. These, after various transformations, with other than laccase enzymes participating, may enrich the soil humus fractions[19].

Unfortunately, the fungal sources of laccase known so far are not convenient, because of necessity to use inducers which are often toxic for the environment, e.g. xylidine. Non-induced cultures usually produce rather small amounts of laccase, not sufficient for preparation on a commercial scale. In this work, we describe the purification and preliminary characterization of a inducible and constitutive forms of laccase secreted by the white rot fungus *Cerrena unicolor*. Although this organism produces both inducible and constitutive laccase when inducer is present in the growing medium, it secretes also a considerable amount of constitutive laccase without addition of any toxic inducer.

2. EXPERIMENTALS

2.1 Fungal strain and culture conditions, determination of laccase activity and protein content

The fungal strain *Cerrena unicolor* (Bull. ex Fr.) Murr. No.139 coming from our collections was maintained at 4℃ on the agar slants. For inoculation the fungal agar plugs (ca. 0.5 cm) were cut and grown in a basal medium based on Czapek Dox and Lindeberg media[2] containing different amounts of Na_2HPO_4. The media pH was adjusted by phosphate to the optimal values for particular fungal cultures. The culture solutions were grown in static conical flasks at 27℃ till the mycelium occupied the whole surface of the liquid. The mycelial mats were collected and homogenized in a Waring Blender.

The shallow stationary cultures, after inoculation with 4% (v/v) of the homogenate, were incubated in 1,000 ml flat (Roux type) flasks with 200 ml medium at 27℃. The solutions were sterilized by filtration through Sterivex-GS 0.2 mm filters (Millipore, Bedford, Massachusetts). The inducer (ferulic acid) was added when the mycelium covered about 1/4 volume of the culture surface in a final concentration of 0.2 mM. Some fungal cultures were done without inducer.

Laccase activity was measured at 20℃ on a Shimadzu Graphic Printer PR-1 spectrophotometer with syringaldazine as substrate, but MES-NaOH buffer utilized by Leonowicz and Grzywnowicz[20] was replaced by 0.1 M citrate - phosphate buffer[21]. To exclude endogenous peroxide, the 10 min. preincubation (stirring) of the enzyme sample with catalase (10 mg/ml) was performed. The activity was expressed in the international units, i.e. in nmol/L or nkat/L, by assuming a molar absorption coefficient[21] of 65,000 for the colored (red) reaction product. It was calculated as follows:

$$Act = \frac{\Delta A_{525} \times \text{total volume(ml)} \times 10^9}{E(\text{syring.}) \times \Delta t \times \text{sample volume(ml)}} \quad \frac{\text{nkat}}{\text{l}}$$

where:

$$kat = \frac{mol}{S}$$

E(syringaldazine) = 65,000

Δt = seconds (= 60)

When the total volume = 1 ml and the sample vol = 0.1 ml, then:

$$Act = \Delta A_{525} \times 2564 \quad \frac{\text{nkat}}{\text{l}}$$

The protein contents both in the culture fluids and in laccase preparations were determined according to Lowry et al. by using bovine serum albumin as a standard[22].

2.2 Preparation of affinity columns, purification of enzyme and electrophoresis

The ConA-Sepharose column was prepared according to Froehner and Eriksson[23]. The commercial preparation of ConA-Sepharose column suspended in 0.1 M acetate buffer pH 6, containing 0.1 M NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, was packed into a column of 15 x 1.5 cm. Before the use, the column was washed with 2 vol. of 0.1 M phosphate buffer, pH 6, and equilibrated with the same buffer. AH-Sepharose 4b coupled to syringaldehyde was used in affinity chromatography because of high affinity of laccase for syringal radical[24].

The coupling procedure was performed as described Paszczynski and Trojanowski,[25] but veratraldehyde was replaced by syringaldehyde (Fig. 1). The beds obtained were suspended in 0.5 M NaCl and a column of 15 x 1.5 cm formed was washed with 2 vol. of 0.1 M phosphate buffer, pH 6, and equilibrated with the same buffer.

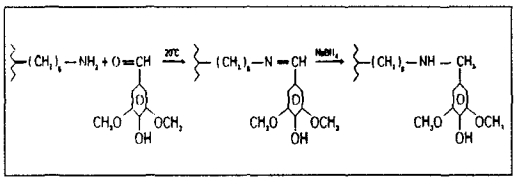

Fig. 1. Scheme of coupling of AH-Sepharose 4B to syringic aldehyde.

For the preliminary purification of laccase the culture with the highest activity level was filtered through Miracloth (Calbiochem). The filtrate was desalted on the Sephadex G-25 (Pharmacia) column, and the enzyme concentrated on the Amicon ultrafiltration system to ca. one tenth of the volume using a filter type PTGC with a pore

size 10,000 NMWL at 4℃. The concentrated preparation was applied onto DEAE-Cellulose 23 SS column (Serva) and eluted by a gradient 0 - 0.35 M Na_2SO_4 in 5 mM citrate- phosphate buffer, pH 6.5. The fractions around the top of the activity (one peak) eluted by NaCl (near 0.2 M) were collected, dialyzed to water and lyophilized.

For high purification of the enzyme, the culture incubated with and without inducer was filtered through Whatman No. 4 filter paper, pooled and concentrated to ca. one tenth of the volume using Millipore equipment Cat. No. PTGCOLM2 (Lot No. N2JMA09A) filter type PTGC with a pore size 10,000 NMWL at 4℃. In some cases, the culture filtrates were saturated with ammonium sulfate at the saturation range 0 - 0.8 and centrifuged (15 min, 15000 x g).

The precipitates were dissolved in 0.1M phosphate buffer pH 6.0. The concentrated (both by ultrafiltration and ammonium sulfate) culture filtrates were dialyzed 7 times to the deionized water and (before the end of dialysis) once to 0.1 M phosphate buffer, pH 6. Then 25 ml portions of such prepared enzyme preparations were applied onto the ConA-Sepharose columns (7 x 1.5 cm), which were then washed with 0.1 M phosphate buffer, pH 6, 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; the DEAE-Sephadex A-50 columns (25 x 1.5 cm) were washed and equilibrated with 0.01 M phosphate buffer, pH 6. The separated laccase-rich fractions eluted successively from the column were dialyzed against 0.1 M phosphate buffer, pH 6, and applied separately onto the columns of AH-sepharose 4B coupled to syringaldehyde. Other proteins were removed from the column by 0.1 M phosphate buffer, pH 6, and then laccase was eluted by 0.5 M ammonium sulphate dissolved in the same buffer, dialyzed against 0.01 M phosphate buffer, pH 6, and kept under nitrogen at 0℃.

The inducible and constitutive forms of laccase were identified by their electrophoretic migration rate on polyacrylamide gel, as described by Leonowicz et al.[26]. Cylindrical as well as slab gels were performed in the Tris/borate system, pH 8.45, according to Leonowicz and Trojanowski[27]. Protein bands on gels were visualized with a Coomassie Brilliant Blue R (Sigma, St. Louis, Missouri), and laccase, after adjustment of pH in gels to 5.5, by the reaction with *p*-phenylenediamine[28].

2.3 Investigation of properties of purified enzyme

Laccase activity was assayed at different temperatures in 0.1 M phosphate buffer, at pH optimum for particular laccase preparations, or at different pH values at 20℃ in 0.1 M McIlvaine (citrate/phosphate) buffer[21]. The rate of syringaldazine oxidation by purified laccase preparations was measured in 0.1 M McIlvaine buffer at pH optimum for particular laccase preparations. The Km values were calculated from the double reciprocal plots[29] using the Enzeftter (Biosoft) software in an IBM-AT computer (Siemens, PCD-2M).

Activity with various phenols was determined polarographically with the use of oxygen electrode according to Leonowicz et al.[26]. Determination of molecular mass was performed by the cationic detergent polyacrylamide- gel electrophoresis using cetyltrimethylammonium bromide (CTAB) according to Marjanen and Ryrie[30].

The sugar content of the purified laccase forms

was examined with a glycan detection kit according to Fukushima and Kirk[31]. It was calculated from the differences in the CTAB electrophoresis determined from molecular weights of laccase forms before and after deglycosylation with hydrogen fluoride[32]. The Cu content was calculated by comparison of UV and visible spectra of the enzyme forms according to the method of Fukushima and Kirk[31] based on the Cu status in the model fungus *Coriolus versicolor*[33,34].

2.4 Chemicals

Coniferyl alcohol, vanillin, veratraldehyde, syringyl aldehyde, catechol, guaiacol, orcinol, 1,4-phenylenediamine dihydrochloride, pentachlorophenol (PCP), N,N,N' N' -tetramethylenediamine (TEMED) and cetyltrimethyl- ammonium bromide (CTAB) as well as caffeic, chlorogenic, ferulic, gallic, protocatechuic, sinapic, syringic thioglycolic vanillic, and veratric acids, were purchased from Fluka A.G. (Buchs, Switzerland). ConA-Sepharose, AH-Sepharose and DEAE-Sephadex A-50 were from Pharmacia (Uppsala, Sweden), acrylamide and N,N' -methylene-bis-acrylamide were from B.D.H. (Poole, England), lauryl sulfate and Coomassie BB from Serva (Heidelberg, Germany), bovine liver catalase and syringaldazine were from Aldrich-Chemie (Steinheim, Germany). LMD Calibration Kit Proteins was obtained from Pharmacia (Uppsala, Sweden) glycan detection kit from Boehringer Mannhaim (Indianapolis, Indiana).

3. RESULTS AND DISCUSSION

Laccase is known to occur in some fungi as constitutive and inducible forms[27,28,35]. An excess of saccharose or glucose in the liquid medium eliminated the spontaneous induction of laccase. These media allow the constitutive production of laccase by fungi, whereas the biosynthesis of the induced enzyme form is repressed by sugar[2].

The extracellular *Cerrena unicolor* laccases were isolated and purified both from non-induced and induced cultures at the peak of activity. Depending on further investigation requirements two approaches of the enzyme purification were applied. Preliminary purification based on ion exchange chromatography on DEAE-Cellulose 23 SS column was simple and gave higher yields. Using this routine method we obtained a relatively high amount of the enzyme preparation, but the laccase forms were not separated. Much higher purification and good separation of the enzyme even into three forms (two constitutives and one inducible) were achieved using two steps of affinity chromatography. The results are presented in Table 1.

Affinity chromatography on ConA-Sepharose was first applied by Froehner and Eriksson[23] for purification of laccase from *Neurospora crassa*: the degree of the final enzyme purification was about 100-fold. Further the enzyme from *Pholiota mutabilis* culture was purified only 50-fold by the same method[24]. These rather poor results could be explained by a non-specific complexation of the laccase center with Concanavalin through the -glycosidic groups of the carbohydrate component[23] of the laccase protein. Since, in addition to laccase, any other glycoproteins occurring in fungi, may be able to bond to Concanavalin. As a result, this procedure separates glycoproteins from other proteins. However, we utilized the specificity of this adsorbent and applied ConA-Sepharose chromatography as the second purification step in addition to fractionation with ammonium sulfate.

The ion-exchange chromatography on DEAE-

Table 1. Isolation and purification of *Cerrena unicolor* laccase forms

Purification step	Activity		Yield %	Purification fold
	Total nkat/vol	Specifictotal/mg protein		
Cerrena unicolor (non-induced culture)				
Filtrate	42675	53	00.0	1.0
Ultrafiltration	38243	191	89.6	3.6
ConA-Sepharose	32347	1970	75.8	37.2
DEAE-Sephadex A-50				
form I	9343	5443	21.9	102.7
form II	8127	4793	19.0	90.4
form III	75	5276	0.2	99.5
Syringyl-AH-Sepharose 4B				
form I	4275	9398	10.0	177.3
form II	3867	6273	9.1	118.3
form III	42	8721	0.1	164.5
Cerrena unicolor (induced culture)				
Filtrate	61784	59	100.0	1.0
Ultrafiltration	58270	202	94.3	3.4
ConA-Sepharose	52276	2127	84.6	36.1
DEAE-Sephadex A-50				
form I	15378	6434	24.9	109.1
form II	13274	6232	21.5	105.6
form III	3428	6927	5.5	117.4
Syringyl-AH-Sepharose 4B				
form I	6234	10743	10.1	182.1
form II	5867	7113	9.4	120.6
form III	1994	10634	3.2	180.2

Sephadex A-50 enabled the separation of the enzyme into inducible and constitutive forms with a simultaneous purification (Table 1). These forms were identified as described in the report by Leonowicz et al.[26]. Forms I and II are constitutive, form III is inducible. The presence of a small amount of an inducible form in the enzyme preparation, coming from the not-inducible culture (see Table 1), may result e.g. from the possible endogenous synthesis of some aromatic compounds, e.g. veratryl alcohol[36] which may be transformed in the culture to laccase inducing veratraldehyde and veratric acid.

The phenomenons of endogenous laccase inducibility were also observed earlier[24,26]. We stated, however, that ferulic acid used in this work as the inducer, is not endogenously produced by *Cerrena unicolor* culture. Anyway, the production of even small amounts of the highly active form III laccase in the non-induced culture is an

interesting point for further investigations.

The last step of the purification procedure consisted to an affinity chromatography based on the specificity of the active site of laccase towards syringic acid. This substrate is intensely oxidized and specifically demethylated by laccase[2]. A high affinity of the support containing syringyl arm towards laccase permitted for high purification of the enzyme forms.

The laccases obtained in the successive steps of purification procedure were subjected to electrophoresis on the polyacrylamide gel. The final preparations of the laccase forms were homogeneous on CTAB polyacrylamide gel electrophoresis (Fig. 2). The pH and temperature optimum of the purified laccase were pH 5.5 and 40 ℃, respectively, which confirmed the results of Bekker et al.[37]. Similar values were found for all the enzyme forms. Michaelis constants obtained with syringaldazine as a hydrogen donor were 73.7 x 10 M, 71.6 x 10 M for the constitutive (I and II) forms respectively, and 16.7 x 10 M, for inducible form III. It indicates higher affinity towards the substrate of the inducible form. Also the higher activity of the inducible forms than the constitutive ones towards all the phenolic substrates tested (Table 2) confirms our earlier suggestion[24,26], that the inducible forms of laccase play an important role in the metabolism of wood-rotting fungi.

Morohoshi et al.[38] and Morohoshi and Haraguchi[39] found that only one of three laccases isolated from *Coriolus versicolor* (synonym *Trametes versicolor*) depolymerized certain lignin compounds, while the other two laccases gave

Table 2. Substrate specificity of the constitutive and inducible forms of laccase from *Cerrena unicolor*

Substrate*	Activity (nmol O_2/min)		
	Constitutive		Inducible
	form I	form II	form III
Coniferyl alcohol	25.3	27.4	53.2
Chlorogenic acid	63.3	62.7	112.9
Gallic acid	132.3	141.7	325.4
Guaiacol	287.8	297.3	683.7
Caffeic acid	356.2	338.9	982.9
Ferulic acid	279.4	296.4	898.5
Orcinol	93.7	95.3	198.2
Pentachlrophenol	11.6	13.8	24.7
Protocatechuic acid	243.2	231.8	452.7
Sinapic acid	325.7	241.2	653.6
Syringic acid	378.9	367.5	692.6
Syringyl aldehyde	243.5	238.0	456.3
Vanillic acid	398.2	379.6	712.8
Vanillin	124.6	119.7	243.2
Veratraldehyde	13.8	11.3	19.2

* The phenolic substrates were added at a concentration of 2 mM to the reaction mixture containing 100 units of laccase activity (cf. Table 1).

mainly polymerized products. Thus the possible differentiation between specificity of particular laccase forms was confirmed also by other authors.

Molecular weights were 65 and 63 kD for constitutive forms I and II respectively, and 59 kD for inducible form III (see Fig. 2). The sugar contents in these forms were 19%, 17% and 15% respectively. It corresponds to the sugar content in the laccase forms isolated from the ligninolytic fungus *Ceriporiopsis subvermispora* (10% and 15% for the constitutive forms named L1 and L2, respectively[31]). Every form of laccase contained 4 mol of copper per protein molecule, as it was demonstrated for the *Coriolus versicolor* laccase[33,34].

Concluding, the laccase forms produced by fungus *Cerrena unicolor*, were isolated and highly purified by affinity chromatography, and some of their properties, besides those in the report by Russian authors[37], were determined. These

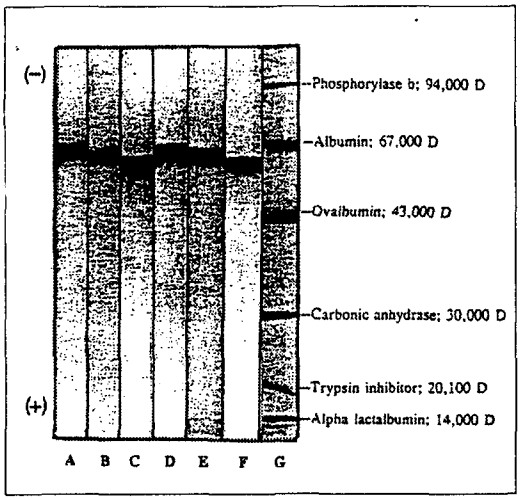

Fig. 2. Polyacrylamide gel elecrophoresis of the purified laccase forms under denaturated conditions. ABC - forms I, II and III, respectively isolated from the non - induced culture, DEF - respectively forms I, II and III isolated from the induced culture, G - LMD Calibration Kit Proteins.

preparations can be applied for some more sophisticated biotechnological methods, e.g. in diagnostics or for analytical investigations requiring high protein purity. However, for many biotechnological reasons not highly purified enzyme isolated from non-induced cultures is sufficient. The preliminary purified, relatively cheap laccase preparation is very stable: kept one year at 4℃ in lyophilized state loses not more than 5% of its initial activity. Instead of laccase preparations even whole *Cerrena unicolor* culture or unrefined culture filtrate could be also used. These are probably the richest sources of fungal laccase known so far.

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