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Purification and Partial Characterization of the Extracellular Laccase from *Ophiostoma novo-ulmi*

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Abstract. *Ophiostoma ulmi* and *O. novo-ulmi*, both causative agents of Dutch elm disease, can be differentiated by their potential to produce constitutive extracellular laccase. The enzyme has been purified from the culture filtrate to apparent electrophoretic homogeneity and has been partially characterized. The laccase was glycosylated and found to have a molecular mass of 79 kDa or 70 kDa by SDS-PAGE and gel filtration, respectively. The pI, determined by chromatofocusing, was 5.1. Syringaldazine, guaiacol, and other typical laccase substrates were oxidized. No oxidation of tyrosine was detected. NaN_3 (0.01%) completely abolished the activity towards 2,6-dimethoxyphenol.

There are two systems of plant pathogen interaction so far examined in which strong evidence has been found for the involvement of extracellular laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) in pathogenesis. One is the chestnut blight fungus *Cryphonectria parasitica* on chestnut, where laccase is thought to be responsible for the detoxification of tannins in the chestnut bark and where the enzyme is downregulated in hypovirulent isolates of the pathogen [12, 16]. The other is the soft rot fungus *Botrytis cinerea* on cucumber, where it has been shown that cucurbitacins (tetracyclic triterpenoids produced by cucumber) protect the plant from infection and at the same time specifically suppress the formation of extracellular fungal laccase [1, 2]. Recently, Pezet and colleagues [14] have found that the laccase of *B. cinerea* is capable of detoxifying stilbene derivatives (phytoalexins produced by grape), thus discovering a new, although speculative function in the interaction between this pathogen and the grape berry plant.

It has been previously shown that *Ophiostoma ulmi* and *O. novo-ulmi*, both vascular wilt pathogens and causative agents of Dutch elm disease, can be differentiated by their potential to produce extracellular laccase [3]. *O. novo-ulmi* is a strong pathogen on native North American and European elms [4, 5] and produces high amounts of laccase. *O. ulmi*, on the contrary, is weakly pathogenic and secretes very low amounts of the enzyme. No direct involvement of laccase in Dutch elm disease pathogenesis has been demonstrated so far, although it may be possible that the enzyme is capable of detoxifying endogenous phenols present in the host or produced in response to infection. This may confer a higher survival to *O. novo-ulmi* during the saprophytic bark phase [3].

In order to gather more detailed knowledge about the laccase protein secreted by *O. novo-ulmi* and also as a prerequisite for subsequent internal microsequencing, we report here the purification and partial biochemical characterization of this enzyme.

Materials and Methods

Organisms and culture conditions. The isolate *O. novo-ulmi* CKT-11 was kindly provided by Clive M. Brasier (Forest Research Station, Alice Holt Lodge, Farnham, Surrey GU 10 4LH, UK). Media and culture conditions were the same as previously described [3].

Laccase assay. Extracellular laccase activity was determined with 2,6-dimethoxyphenol (DMOP), as previously described [3, 18].

Protein determination. Protein concentrations were determined by the method of Bradford [7], with bovine serum albumin (Fluka) as standard.

Enzyme purification. The purification procedure except the Superdex 75 (Pharmacia) gel filtration and the Diethylaminoethyl (DEAE) ion exchange chromatography steps was carried out at 4°C. The extracellular laccase of *O. novo-ulmi* CKT-11 grown on glucose (500 ml of medium in 1-L Erlenmeyer flasks) was purified from the culture filtrate

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Table 1. Purification of extracellular laccase from shaken glucose cultures of *Ophiostoma novo-ulmi* CKT-11

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Crude enzyme (culture filtrate)	7500	70.7	2450	34.9	100	1
Concentrated ultrafiltrate	320	14.8	1160	225	47.4	6.4
Laccase after S-Cartridge	3	3.33	750	317	30.6	9.8
Laccase after Superdex gel filtration	3	0.51	320	634	13.1	18.2
Laccase after DEAE-TSK ion exchanger	3	0.062	72	1161	2.9	33.3

collected after 62 h. 7.5 L of culture medium was first filtered through Miracloth (Calbiochem) and filter paper (Schleicher & Schuell); the still turbid filtrate (containing yeast cells) was then clarified by centrifugation for 10 min at 5000 *g*. The supernatant was concentrated to about 300 ml with an ultrafiltration cassette (Filtron, MW exclusion size = 10 kDa) and dialyzed against sodium acetate 50 mM (pH 3.8). The concentrate was centrifuged for 10 min at 10,000 *g* and applied to an S-Cartridge column (6 ml bed volume, Biorad) equilibrated in sodium acetate 50 mM, pH 3.8. The cartridge was washed with three bed volumes of buffer and then eluted with sodium acetate 50 mM (pH 3.8) containing 250 mM NaCl. Active fractions were combined and were directly applied to a gel filtration system (Superdex 75, 16/60, Pharmacia) equilibrated in 50 mM potassium phosphate (pH 6.0, 0.15 M NaCl). The combined active fractions were concentrated by membrane ultrafiltration (PM10, Amicon), dialyzed against piperazine 20 mM (pH 6.0), and applied to a DEAE ion exchange column (bed volume 1 ml, LKB). The column was washed with ten bed volumes and then eluted by a linear gradient of 0–200 mM NaCl. The active fractions were collected, concentrated, and dialyzed against potassium phosphate 20 mM (pH 6.0) by membrane ultrafiltration, then stored at −20°C for several weeks without loss of activity.

Gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli [13], and the gels were stained with Coomassie blue (Pharmacia). Glycoprotein staining after native PAGE with Schiff's base was performed as described by Zacharius et al. [20].

Estimation of pI by chromatofocusing. The pI of the purified laccase was determined by loading an aliquot of the pure enzyme (approx. 5 µg) on a MonoP (5/20, Pharmacia) chromatofocusing column equilibrated with 10 ml 25 mM of histidine HCl (pH 6.2), and the gradient between pH 6.2 and 4.0 was generated by applying 45 ml of 12.5% (vol/vol) Polybuffer 74 (Pharmacia) in water adjusted to pH 4.0 with HCl.

Substrate specificity. All substrates (cf. Table 2) were tested at a final concentration of 2 mM in McIlvaine citrate-phosphate buffer with pH values ranging from 2.2 to 7.6 at 25°C. For 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), DMOP and syringaldazine, the initial increase in optical density at the indicated wave length was monitored. With all other substrates (cf. Results), the reaction mixtures had to be incubated for a longer time period.

Results

Enzyme purification. A summary of the purification procedure is given in Table 1. Four purification steps were required to separate the laccase from other extracellular proteins, polysaccharides, and pigments. After ultrafiltration, the sample was highly viscous and dark-colored. A second step (cation exchange chromatography) yielded a highly concentrated, yellow enzyme sample which apparently was still heavily contaminated with extracellular polysaccharides (EPS). This was confirmed by measuring the rather low A_{280}/A_{260} ratio (= 1.38). A similar situation has been observed for the laccase of *C. parasitica* (D. Rigling, pers. communication). The EPS were subsequently removed by Superdex gel-filtration chromatography, and the estimated native molecular weight of the enzyme was 70 kDa as judged from the eluting position from this column (Fig. 1a). At this stage, the sample was colorless, but still contained other proteins as judged from SDS-PAGE (data not shown). Finally, the laccase could be recovered as a single symmetrical peak from a DEAE anion-exchange column (Fig. 1b).

Properties of the laccase. The enzyme was homogeneous as judged by SDS-PAGE, but the molecular weight determined by this method was 79 kDa (Fig. 2). The pI of laccase was estimated by chromatofocusing to be 5.1 (data not shown). The specific activity was 1161 U mg^{-1} as indicated in Table 1. Several typical laccase substrates were oxidized by the purified enzyme as indicated by an intense increase of the optical density measured at the appropriate wave length (Table 2). Tyrosine was not oxidized. A total inhibition of the activity with DMOP was observed when 0.01% NaN_3 was added. With DMOP and ABTS as substrates, the pH optimum for activity of the purified laccase was 2.8, whereas with syringaldazine, gallic acid, and guaiacol it was 6.0. Native polyacrylamide gels (10%) treated with Schiff's base stained the laccase band, suggesting a glycoprotein nature of the enzyme (results not given).

Discussion

Our work extends the recent report on the differential production of extracellular laccase by the two Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi* [3]. The main difficulty while purifying the extracellular laccase of *O. novo-ulmi* was the removal of the fungal EPS. However, the molecular weight of these polysaccharides was sufficiently high to allow their elimination by size exclusion chromatography. Indeed, a prominent yellow-colored peak probably containing the EPS eluted

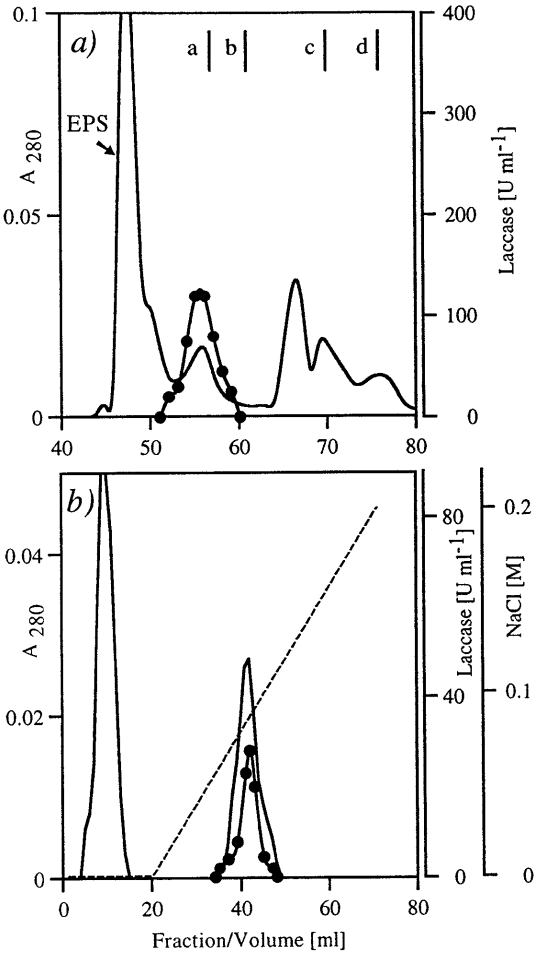

Fig. 1. Superdex gel filtration and DEAE anion exchange chromatography of laccase of *O. novo-ulmi*. (●) Laccase activity. (a) Separation of EPS (arrow) from laccase by gel filtration. Vertical bars on the top represent molecular size markers: a) Albumin (67 kDa), b) Ovalbumin (43 kDa), c) Chymotrypsinogen (25 kDa), d) Ribonuclease (13.7 kDa). (b) DEAE anion exchange chromatography separating the pure laccase from other, nonbinding proteins. The dotted line represents the NaCl gradient.

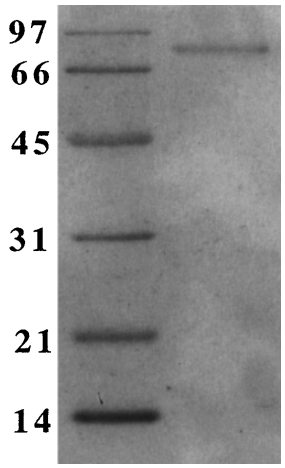

Fig. 2. SDS-PAGE of the purified extracellular laccase. Molecular mass markers are shown in the left lane.

Table 2. Substrate specificity of extracellular laccase from *O. novo-ulmi.* Each test was performed with 0.15 U of the pure enzyme

Substrate (2 mM)	Optimal pH	Wavelength (nm)	ΔOD min^{-1} mg^{-1}
DMOP	2.8	468	1160
ABTS	2.8	610	339
Syringaldazine	6.0	525	2700
Guaiacol	6.0	470	59
Gallic acid	6.0	470	[a]
Tyrosine		460	0
DMOP + NaN_3 (0.01%)		468	0

[a] A strong color reaction was visible upon incubation overnight.

with the void volume. The molecular mass estimates of 79 kDa by SDS-PAGE and of 70 kDa by gel filtration are within the molecular weight ranges (55,000–85,000 Da) reported for the laccases of e.g., *Botrytis cinerea*, *Fomes annosus*, or *Trametes versicolor*, [6, 10]. Additionally, the close values determined by the two different methods indicate a monomeric nature of the enzyme.

The other properties so far demonstrated for *O. novo-ulmi* laccase are also not atypical of fungal extracellular laccases. For instance, Eggert, Temp and Eriksson [9] showed that the pI of laccase II from *Pycnoporus cinnabarinus* was 4.8. Rigling and Van Alfen [17] and Fukushima and Kirk [11] found very acidic pH optima for the laccase activities of *Cryphonectria parasitica* with DMOP (2.5) and *Ceriporiopsis subvermispora* with ABTS (3.0), respectively. In addition, all fungal laccases so far described are glycosylated [15, 19]. Therefore, our findings suggest that the *O. novo-ulmi* laccase, like all laccases characterized, can confidently be expected to be a copper-containing glycoprotein. The fact that the pure laccase sample was colorless and not blue, like most fungal laccases, is probably owing to the low protein concentration (20 μg/ml). This explanation has also been claimed for the pure laccase of *Schizophyllum commune*, which was also colorless [8].

The purification of laccase is a first step towards its detailed characterization (cloning and determination of the nucleotide sequence of the respective cDNA). As a next step, since the N-terminus of the enzyme was blocked, we plan to obtain an internal amino acid sequence from the fragmented protein generated by proteolytic digestion. This will then hopefully allow the cloning of the respective gene and give a clue to the function of the enzyme during pathogenesis of Dutch elm disease.

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Literature Cited

1. Bar-Nun N, Mayer AM (1989) Cucurbitacins-repressors of induction of laccase formation. Phytochemistry 28:1369–1371
2. Bar-Nun N, Mayer AM (1990) Cucurbitacins protect cucumber tissue against infection by *Botrytis cinerea*. Phytochemistry 29:787–791
3. Binz T, Canevascini G (1996) Differential production of extracellular laccase by the Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi*. Mycol Res 100:1060–1064
4. Brasier CM (1986) A comparison of pathogenicity and cultural characteristics on the EAN and NAN subgroups of *Ophiostoma ulmi*. Trans Br Mycol Soc 83:1–13
5. Brasier CM (1991) *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. Mycopathologia 115:151–161
6. Bollag JM, Leonowicz A (1984) Comparative studies of extracellular fungal laccases. Appl Environ Microbiol 48:849–854
7. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
8. DeVries OMH, Kooistra WHCF, Wessels JGH (1986) Formation of an extracellular laccase by *Schizophyllum commune* dikaryon. J Gen Microbiol 132:2817–2826
9. Eggert C, Temp U, Eriksson KEL (1996) The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. Appl Environ Microbiol 62:1151–1158
10. Eriksson KEL, Blanchette RA, Ander P (1990) Biodegradation of lignin. Berlin: Springer-Verlag
11. Fukushima Y, Kirk TK (1995) Laccase component of the *Ceriporiopsis subvermispora* lignin-degrading system. Appl Environ Microbiol 61:872–876
12. Kim DH, Rigling D, Zhang L, Van Alfen NK (1995) A new extracellular laccase of *Cryphonectria parasitica* is revealed by deletion of Lac1. Mol Plant-Microbe Interact 8:259–266
13. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
14. Pezet R, Pont V, Hoang-Van K (1992) Enzymatic detoxification of stilbenes by *Botrytis cinerea* and inhibition by grape berries proanthrocyanidins. In: Verhoeff K, Malathrakis NE, Williamson B (eds) Recent Advances in Botrytis research. Wageningen: Pudoc Scientific Publishers, pp. 87–92
15. Rehman AU, Thurston CF (1992) Purification of laccase I from *Armillaria mellea*. J Gen Microbiol 138:1251–1257
16. Rigling D, Van Alfen NK (1991) Regulation of laccase biosynthesis in the plant-pathogenic fungus *Cryphonectria parasitica* by double-stranded RNA. J Bacteriol 173:8000–8003
17. Rigling D, Van Alfen NK (1993) Extra- and intracellular laccases of the chestnut blight fungus, *Cryphonectria parasitica*. Appl Environ Microbiol 59:3634–3639
18. Rigling D, Heiniger U, Hohl HR (1989) Reduction of laccase activity in dsRNA-containing hypovirulent strains of *Cryphonectria* (*Endothia*) *parasitica*. Phytopathology 79:219–223
19. Thurston CF (1994) The structure and function of fungal laccases. Microbiology 140:19–26
20. Zacharius RM, Zell TE, Morrison JH, Woodlock JJ (1969) Glycoprotein staining following electrophoresis in acrylamide gels. Anal Biochem 30:148–152