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Short contribution

Purification and characterization of laccase from *Monocillium indicum* Saxena

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Summary. An ascomycete *Monocillium indicum* Saxena producing extracellular laccase was isolated. The culture filtrate on native polyacrylamide gel electrophoresis (PAGE) revealed four bands of activity, one of which was a major one. The major laccase band, a glycoprotein, was purified and characterized. Gel filtration chromatography showed that the relative molecular weight (M_r) of laccase was 100000. On sodium dodecyl sulphate (SDS)-PAGE the major laccase band further resolved into three proteins of M_r 72000, 56000 and 24000. The enzyme had a pH optimum of 3.0 and was active on a number of *o*-phenols and aromatic acids. The 72000 M_r protein was found to share common immunological properties with laccases of *Coriolus versicolor*, *Agaricus bisporus* and lignin peroxidase of *Phanerochaete chrysosporium*.

Introduction

Lignin is the most abundant renewable aromatic biopolymer. The degradation of lignin is a very slow process and still incompletely understood. The white-rot basidiomycetes are the most efficient lignin degraders and so far the most widely studied. The enzymes implicated in lignin degradation are: lignin peroxidase (LiP), which catalyses the oxidation of both phenolic and non-phenolic units; manganese-dependent peroxidase (Mn-P) and laccase, which oxidize phenolic compounds to give phenoxy radicals and quinones; glucose oxidase and glyoxal oxidase for H_2O_2 production; cellobiose-quinone oxidoreductase for quinone reduction (Kirk and Farrell 1987).

The ascomycetes have not been a focus for lignin degradation studies as much as the white-rot basidiomycetes. Clearly there is a need to study degradation of lignin and the responsible enzymes in ascomycetes. In this paper we report for the first time the characterization of a laccase showing peroxidative activity from an ascomycete, *Monocillium indicum* Saxena.

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Materials and methods

Culture. M. indicum Saxena was isolated from a paper-mill soil sample in our laboratory and identified by the International Mycological Institute, Surrey, UK (IMI Herbarium no. 329787). The fungus was maintained on Sabouraud's agar.

Culture conditions. For laccase production a modified medium previously described by Asther et al. (1987) was used. The medium contained glucose (1%), was buffered with 20 mM phthalate buffer, pH 5.0, and did not contain oleic acid and veratryl alcohol. A homogenized inoculum was used at 20% (v/v) concentration. The organism was cultivated in 250-ml conical flasks containing 20 ml medium and incubated at 28° C (±2° C) under static conditions for 6 days and the extracellular culture filtrate used for assaying laccase after removal of mycelium.

Laccase activity. Laccase was assayed using 2.8 ml of 0.34 mM *o*-dianisidine in 0.1 M sodium acetate buffer, pH 5.0 (buffer A). The reaction was initiated by the addition of 200 µl of the enzyme and the increase in absorbance was monitored at 460 nm on a spectrophotometer. One unit of laccase is defined as the amount of enzyme required to cause a change in absorbance of 1.0 per minute at 28° C.

Enzyme purification. $(NH_4)_2SO_4$ precipitates of the extracellular culture filtrate were dissolved in buffer A, dialysed and the enzyme was further purified by preparative native polyacrylamide gel electrophoresis (PAGE) (8%, w/v).

Enzyme characterization. The relative molecular mass (M_r) of the major laccase band as purified by preparative PAGE was determined by fast protein liquid chromatography (FPLC) (Pharmacia, Uppsala, Sweden), using a Sephacryl-S-200 column (48 × 1.3 cm) equilibrated with buffer A and calibrated with standard M_r proteins (MW-GF-200 kit, Sigma, St. Louis, USA) with a flow rate of 1.0 ml/min.

Electrophoresis, native and sodium dodecyl sulphate (SDS), was performed as described by Laemmli (1970) and Sigma SDS-6H standards were used. Lithium dodecyl sulphate (LDS) gels were run by the method of Evans (1984). Protein bands were stained with Coomassie brilliant blue dye. Laccase on native and preparative PAGE was visualized by activity-staining with *o*-dianisidine. Glycoprotein staining was carried out by the method of Za-

charias et al. (1969). Peroxidase activity on LDS gels was visualized by the method of Evans (1984).

The major laccase band of *M. indicum* was immunoblotted with specific antibodies to LiP of *Phanerochaete chrysosporium* and laccase of *Agaricus bisporus* and *Coriolus versicolor* (Gallagher 1989; Gallagher et al. 1989) after separation on SDS-PAGE and transferring to nitrocellulose paper as described in Bio-Rad Immuno Blot (Hertfordshire, UK).

Activity of laccase towards a variety of substrates was determined polarographically as described by Dubernet et al. (1977) using 1.0 mM substrate in buffer A in an oxygraph (Gilson, Villiers-le-Bel, France). Activities were calculated as ppm O_2 consumed/min per milligram protein. Protein was determined by the Bio-Rad method (Bradford 1976).

Results and discussion

A laccase-producing ascomycete was isolated in our laboratory on the basis of a positive Bavandamm reaction indicating that it is a white-rot fungus (Nobles 1965). The extracellular culture filtrate of *M. indicum*, when electrophoresed on native PAGE, resolved into one major band and three minor bands of laccase (Fig. 1C). The purification procedure of the major laccase band is summarized in Table 1. Purification resulted in a 1.9-fold increase in specific activity with 21.1% recovery. Figure 1B shows that there were comparatively few proteins in the culture filtrate, thus major increases in specific activities were not expected during purification. Staining of the gel with Schiff's reagent showed that the major laccase band was a glycoprotein (Fig. 1A).

The approximate M_r of the purified major laccase band as determined by gel filtration FPLC was 100000. On SDS-PAGE the purified protein resolved into a major component of apparent M_r 72000 and two minor components of apparent M_r 56000 and 24000, respectively (Fig. 1D). Similar examples of fungal laccases be-

Table 1. Purification of extracellular laccase from *Monocillium indicum* Saxena

Purification step	Total protein (mg)	Specific activity	Recovery (%)	Purity (fold)
Culture filtrate	26.64	34.58	100.0	1.0
$(NH_4)_2SO_4$ (50–85%)	7.44	65.19	52.6	1.8
Preparative PAGE	3.00	67.00	21.1	1.9

PAGE, polyacrylamide gel electrophoresis

Table 2. Characteristics of the purified major laccase band from *M. indicum* Saxena

Property	Observation
pH optimum	3.0 (≅5.0)
Temperature stability	Stable up to 60° C (10 min)
Half-life at 75° C	10 min
K_m	0.025 mM (o-dianisidine)
V_{max}	6.25 units (o-dianisidine)
Inhibition (1.0 mM)	96–100% (cyanide, azide, thioglycollic acid, diethyldithiocarbamate)
Immunological properties	72000 relative molecular weight protein cross-reacted with antibodies against laccase of *Agaricus bisporus*, *Coriolus versicolor* and LiP of *Phanerochaete chrysosporium*
Substrate specificity	204% (ascorbic acid) 50–100% (*o*-dianisidine, catechol, guaiacol, caffeic, vanillic, ferulic, syringic and protocatechuic acids) 15–35% (*p*-anisidine, syringaldizine, *p*-hydroxybenzoic acid and phloroglucinol)

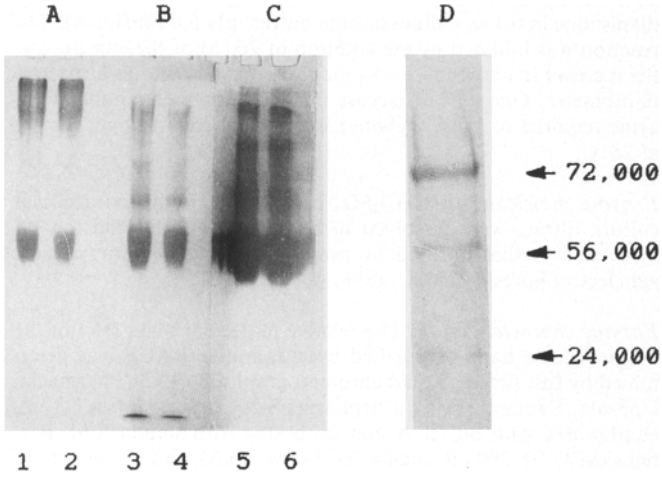

Fig. 1A–D. Polyacrylamide gel electrophoresis (PAGE) of the proteins produced by *Monocillium indicum*. **A** Glycoprotein staining. **B** Protein staining. **C** Laccase staining (activity staining). Lanes 1, 3, and 5 contain 50 µg protein and lanes 2, 4, and 6 contain 30 µg protein. **D** Sodium dodecyl sulphate (SDS)-PAGE of the major laccase band. Relative molecular weights were calculated from Sigma SDS-6H standards and are indicated by *arrows*

having as a single protein in some separation system but as multiple forms in others have been reported (Wood 1980; Kurtz and Champe 1982).

The kinetic and other properties of the major laccase band are shown in Table 2. The laccase was active in the acidic range of pH 2.0–6.0, showing two distinct peaks, one at pH 3.0 and another at pH 5.0, the optimum being pH 3.0 with *o*-dianisidine as substrate. A bimodel distribution of pH optima for the substrate *p*-phenylenediamine has been reported for the laccase of *A. bisporus* (Wood 1980). The pH optima for laccase differs depending on the substrate oxidized (Mayer and Harel 1979). Thermostability of the enzyme was checked by maintaining the protein at temperatures ranging from 20–80° C for 10 min. The enzyme was stable at temperatures up to 60° C, but was completely inactivated at 80° C under these conditions. The temperature required for 50% inactivation of the enzyme in 10 min was 75° C. The inhibition pattern of the laccase from *M. indicum* corresponded with that of other laccases (Dubernet et al. 1977). It was inhibited by cyanide and azide, which

are classical inhibitors of metal-containing oxidases. Inhibition by diethyl dithiocarbamate and thioglycollic acid indicate that it contains copper. The enzyme was active on a wide range of phenolic substrates. More than 70% oxidation of *ortho*-substituted compounds was obtained with the pure laccase, whereas *para* compounds and the *m*-phenol phloroglucinol were oxidized at a relatively low rate.

The purified major laccase band after SDS-PAGE was immunoblotted with polyclonal antibodies raised against laccase of *C. versicolor*, *A. bisporus* and LiP of *P. chrysosporium*. The reaction of the 72000 M_r protein of *M. indicum* with antibodies of laccase from *C. versicolor* and *A. bisporus* shows that these proteins have similar immunological epitopes. The interesting finding was, however, the fact that the laccase reacted with antibodies of LiP from *P. chrysosporium*. Serological similarities have been found between the LiP of *Phlebia* and those of *Phanerochaete* (Kantelinen et al. 1988), but not between LiP and Mn-P in *P. chrysosporium* (Leisola et al. 1987) or oxidases and peroxidases in *Phlebia radiata* (Niku-Paavola et al. 1988). This suggests apparent specificity of the antibodies. Southern hybridization of the DNA from *M. indicum* with the probe for LiP gene from *Phanerochaete chrysosporium* gave a positive hybridization signal, suggesting the presence of DNA sequences homologous to the LiP gene of *P. chrysosporium* (data not shown). However, we were unable to detect any LiP activity with the pure laccase enzyme. Staining of the major laccase band for peroxidase reaction after electrophoresis on LDS gels gave a positive reaction. In the presence of H_2O_2, the major laccase band exhibited a 1.7-fold increase in activity when the assay was performed after the addition of catalase, suggesting the presence of associated peroxidative activity.

Based on our findings, it appears that the laccase (major laccase band on native PAGE) of *M. indicum* can also catalyse peroxidative-type reactions. Whether the peroxidase is of LiP, Mn-P or general fungal peroxidase type needs further investigation. It would be interesting to compare the extent of homology between LiP genes of *P. chrysosporium* and fungal peroxidase genes with those of *M. indicum*.

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