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LACCASE FROM PLEUROTUS OSTREATUS

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Summary

Laccase was purified from culture broth of Pleurotus ostreatus mycelium. The enzyme was a single protein of M_r 59000, pI 2.9 and was active on o-diphenyl substrates. Amino acid composition and N-terminal sequence (15 residues) were determined. Polyclonal anti-laccase antibodies were obtained.

Introduction

The possibility of using waste lignin in the production of biomass and of useful chemicals appears very attractive. Lignin is degraded preeminently by phenol oxidases which are produced by basidiomycetous fungi as extracellular enzymes. Among these, laccase (EC 1.14.18.1) has received more attention; however its role in lignin biodegradation is not established unequivocally (Ishihara, 1980). It would improve understanding of the action mechanism of laccase and of its potentiality in biotechnological processes to have larger amounts of the enzyme available. One approach to this is the isolation of the gene coding for laccase and the modification of its expression. As a preliminary part of this project, we have purified the extracellular laccase of Pleurotus ostreatus and analysed some essential molecular and catalytic properties of the enzyme. We also found growth conditions in which the mycelium does not express laccase activity.

Experimental procedures

Growth of mycelium: Pleurotus ostreatus (strain 3004 darmycell) mycelium was maintained on agar-potato dextrose (Difco Laboratories, Detroit, MI) plates at 4 °C and grown in the same medium without agar, with or without 0.5% yeast extract (Difco). The cultures (400 ml broth in 1 l flasks) were grown at 30 °C either static or shaken at 100 rev/min. Culture broth was recovered after filtration on filter paper and then on 0.45 μm membrane. To produce radioactive proteins, 1 μCi/ml [^{35}S]-methionine (specific activity)1000 Ci/mmol) was added to the culture broth. Triplicate samples of broth were removed at intervals to follow protein, as radioactivity in the trichloroacetate-precipitable fraction, and laccase secretion.

Electrophoresis: polyacrylamide (10 %) slab gel electrophoresis in 0.1% sodium dodecylsulphate (SDS-PAGE) was carried out as described by Laemmli (1970). The treatment of the gels for fluorography was performed according to Bonner and Laskey (1974), using Kodak-X-Omat

XRP-1 films. Western blots of SDS-PAGE were immunostained as described by Jackson and Thompson (1984), using anti-laccase rabbit IgG and horse radish peroxidase-coupled goat anti-rabbit IgG. Electrofocusing in the pH range 2.5-6.0 was performed on a polyacrylamide-gel slab in a Multiphor apparatus from LKB (Stockholm, Sweden) following the manufacturer's instructions.

Laccase purification: was carried out at 4 °C. Solid ammonium sulphate up to 80% saturation was added to filtered broth (1 l) of cultures grown with yeast extract. The precipitate was collected by centrifugation (15,000 g, 20 min), dissolved in 50 mM sodium phosphate buffer, pH 6.0, and extensively dialysed against the same buffer. The protein was concentrated by ultrafiltration on an Amicon PM-10 membrane and loaded on a DEAE Sepharose (Pharmacia, Uppsala, Sweden) column (1.5 cm x 20 cm) equilibrated with the phosphate buffer. After washing with the equilibration buffer (200 ml), a linear gradient (300 ml) of NaCl 0-0.2 M was applied at a flow rate of 10 ml/h. Eluate was monitored for absorbance at 280 nm and laccase activity. Active fractions were pooled, dialysed against the phosphate buffer and concentrated by ultrafiltration.

The protein sample was loaded on a Con-A Sepharose (Pharmacia) column (1 cm x 8 cm) equilibrated with the phosphate buffer; after washing, laccase was recovered by eluting with the phosphate buffer containing 0.5 M methyl-α-glucopyranoside.

Protein and enzyme determination: protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories Richmond, CA), with bovine serum albumin as standard (Bradford, 1976).

Laccase activity was assayed at 30 °C by monitoring absorbance at 465 nm in a reaction mixture (3 ml) containing 10 mM guaiacol, 50 mM sodium phosphate buffer, pH 6.0, and the enzyme. One unit of laccase activity is the amount of enzyme producing an increase of absorbance of 1.0 in one minute.

Enzyme characterization: amino acid composition was determined by hydrolysing protein samples (40 g) in vacuo at 110 °C for 24, 48, and 72 h in 6 M HCl and analysing them on a Beckman 119 CL apparatus. Half-cystine residues were determined as cysteic acid.

N-terminal sequence of the laccase (1.4 nmols) was determined on a Beckman 890/C sequencer modified with a cold trap using a 200 mM quadrol, double-coupling, single-cleavage program. Polybrene (Pierce, Rockford, IL) in 10 mM NaCl was used as carrier. PTH-aminoacids were analysed by HPLC as already described (Pucci et al., 1983).

Gel-filtration chromatography was performed on a TSK G 3000 column (0.75 cm x 60 cm; LKB) using a FPLC apparatus from Pharmacia. The column was eluted with 20 mM sodium phosphate buffer, pH 7.0, at a flow rate of 0.3 ml/min.

Anti-laccase antibodies were prepared as already described (Sannia et al., 1982).

Results and discussion

Laccase production: Pleurotus ostreatus mycelium was grown in

potato-dextrose broth in the presence and absence of yeast extract. Yeast extract enhanced the growth rate and induced laccase secretion with the expression of a different population of secreted proteins as observed by SDS-PAGE (Fig.1A). Immunostaining of a Western blot of the proteins secreted in the presence of yeast extract showed a protein specifically recognized by anti-laccase antibodies, not present among those secreted in the absence of yeast extract (Fig. 1B).

Laccase purification: after purification (Table 1) a single homogeneous protein was obtained with a 12% yield and a purification of 27-fold. Chromatography on ConA-Sepharose, carried out mainly to remove phenolic contaminants (Luna et al., 1983), indicated for the glycoprotein nature of the enzyme.

Table 1. Purification of laccase from Pleurotus ostreatus.

purification step	protein (mg)	activity (U)	specific activity (U/mg)	yield (%)
culture broth	38	300	7.9	100
ammonium sulphate	5	275	55	92
DEAE-Sepharose chromatography	0.3	53	177	18
ConA-Sepharose chromatography	0.17	36	212	12

Properties: P.ostreatus laccase is an o-diphenyl oxidase, utilizing optimally as substrates guaiacol and pyrocatechol, being inactive on hydroquinone, resorcinol and tyrosine.

As determined by SDS-PAGE, laccase had a Mr of about 59000; molecular-sieve chromatography in non-dissociating conditions suggested its monomeric nature. The pI was 2.9 as determined by electrofocusing on polyacrylamide gel. The amino acid composition, expressed as mols of each amino acid per 59000 g of protein, was:Asx 79; Thr 42; Ser 38; Glx 32; Pro 45; Gly 41; Ala 55; Cys 8; Val 42; Met 5; Ile 32; Leu 38; Tyr 15; Phe 25; His 16; Lys 10; Arg 27; tryptophan not determined. Cysteine was determined as cysteic acid, after performic acid oxidation of the protein.

The N-terminal sequence of the enzyme was:

5 10 15
Ala-Ile-Gly-Ser-Ala-Gly-Asn-Thr-Tyr-Ile-Val-Asn-Gln-Asp-Val-

The selection of laccase-coding clones by anti-laccase antibodies and/or synthetic oligonucleotides, constructed against this N-terminal sequence, is now in progress.

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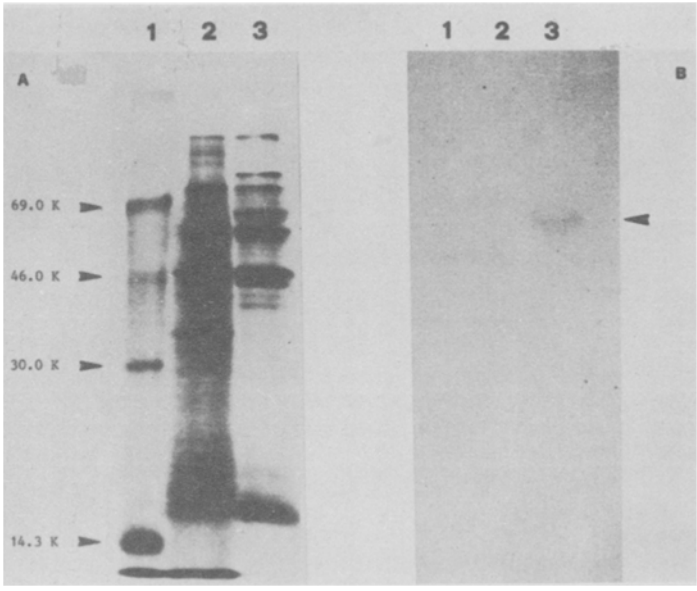

Fig. 1. Fluorography (A) and immunostaining (B) of an SDS-PAGE of proteins secreted by Pleurotus ostreatus. Lane 1: molecular weight markers; lane 2: proteins secreted in the absence of yeast extract; lane 3: proteins secreted in the presence of yeast extract.