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A novel combination of prosthetic groups in a fungal laccase; PQQ and two copper atoms

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Extracellular laccase (benzenediol:oxygen oxidoreductase EC 1.10.3.2) from the lignin-degrading fungus, *Phlebia radiata*, was shown to contain a novel combination of electron carriers as its prosthetic groups. In addition to two copper atoms per enzyme molecule, one molecule of PQQ was included as a cofactor. The EPR spectrum exhibits features of type 1 and type 2 copper atoms. In the enzymatic reaction 4 molecules of lignin model compound, coniferyl alcohol, are oxidized per molecule of oxygen reduced to water. During the reaction coniferyl alcohol is transformed to dilignols.

Laccase, PQQ, Copper enzyme; White rot fungus, Lignin degradation

1. INTRODUCTION

Extracellular laccase (EC 1.10.3.2), a blue copper enzyme, is commonly produced by wood-degrading white rot fungi. This enzyme is able to oxidize compounds containing phenolic hydroxyl groups, including lignin model compounds, and is thus considered to have a role in lignin biodegradation [1]. The best-characterized fungal laccase is from *Polyporus versicolor*. It contains 4 copper atoms per protein molecule [2], whereas laccases with only two copper atoms have been isolated from *Agaricus bisporus* [3] and *Schizophyllum commune* [4].

The white rot fungus *Phlebia radiata* secretes 4 peroxidases and a copper-containing laccase [5,6]. In this work we present the characterization of the prosthetic groups in *Phlebia* laccase, the analysis of copper and the detection of a pyrroloquinoline quinone, PQQ, recently detected in many oxidoreductases [7].

2. MATERIALS AND METHODS

2.1. *Enzyme production and purification*

The organism used was *Phlebia radiata* ATCC 64658. Laccase was produced using a semi-continuous cultivation technique [8]. Purification was performed as described earlier [5], but scaling up to 16 litres of culture filtrate as starting material. Laccase activity was determined using ABTS-stain as substrate [5]. Homogeneity of the purified preparation was confirmed by SDS- and IEF-electrophoresis [6]. Enzyme amount was calculated on the basis of the amino acid sequence of *Phlebia* laccase (M. Saloheimo, VTT, unpublished). Laccase was hydrolysed with 6 M HCl at 110°C for 22 h and the amino acids liberated were analysed on a Waters ion exchanger (P/N 8002). Detection was with *o*-phthaldialdehyde reagent.

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Abbreviations: PQQ, pyrroloquinoline quinone; EPR, electron paramagnetic resonance; ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); EDTA, ethylenediaminetetraacetic acid; AAS, atomic absorption spectrometry; GLC-MS, gas liquid chromatography-mass spectrometry; SDS, sodium dodecyl sulphate; IEF, isoelectric focusing

2.2. *Copper content and EPR spectroscopy*

The purified enzyme was concentrated by ultrafiltration (Centricon 10, Amicon). For control purposes, a part of the sample was dialysed against 50 mM sodium acetate buffer, pH 5.5, containing 1 mM EDTA. Copper content was determined by atomic absorption spectrometry using the graphite furnace technique and checking with standard addition (PE/5000 Zeeman, HGA-400, AS-40). EPR spectra were recorded with a Bruker ESP 300 X-band spectrometer using the following instrumental settings: modulation frequency, 100 kHz; modulation amplitude, 0.99 mT; sweep time/scan, 168, s; sweep width, 0.10 T (1000 G); microwave frequency, 9.44 GHz; microwave power, 1.00 mW. Sample temperature (12 K) was maintained with an Oxford Instruments ESR-900 cryostat system. The spectrum presented in a sum of four scans. A baseline obtained with a water-filled sample tube in the cavity was subtracted from the raw spectra.

2.3. *Determination of PQQ*

PQQ was extracted from purified, lyophilized laccase (62.5 nmol) by the hexanol procedure [9]. The 4-hydroxy-5-hexoxy-PQ derivative obtained was quantified on the basis of absorbance at 318 nm using $\epsilon = 39\,000\ M^{-1}\ cm^{-1}$. Homogeneity and identity were checked by HPLC using an authentic derivative of PQQ as reference. For further confirmation the derivative was converted to PQQ by oxidation with cerium salt [9] and analysed using a biological assay [10].

2.4. *Enzymic reactions*

Coniferyl alcohol was used as electron donor. The reaction mixture contained 0.1 mM coniferyl alcohol (Fluka) and 0.01 nmol enzyme/ml 25 mM sodium lactate, 25 mM sodium succinate buffer at

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pH 4.0. The reaction was followed spectrophotometrically (Shimadzu UV-260) at room temperature by monitoring oxidation of coniferyl alcohol at 263 nm using $\epsilon = 13\,400\ M^{-1} \cdot cm^{-1}$ and measuring the reduction of O_2 using an oxygen electrode (Orion model 97-08). After the reaction 200 U of catalase (Sigma) was added to detect the presence of H_2O_2. For GLC-MS, reaction products were extracted into chloroform from a separate reaction mixture after 10 s incubation and analysed as their trimethylsilyl derivatives [11].

3. RESULTS AND DISCUSSION

The purified and concentrated homogeneous preparation contained 46.5 nmol/ml laccase on the basis of amino acid analysis. The amount of copper analysed by AAS was 100 nmol/ml. These results correspond to 2.1 copper atoms per laccase molecule.

The g_{II} region EPR spectrum (Fig. 1) shows a superposition of one narrowly spaced and one widely spaced signal, i.e. signals from type 1 and 2 coppers. The low-field line above 2700 G originates only from the type 2 copper, whereas both coppers contribute to the features at 2900–3200 G. This is analogous to the spectrum of *Polyporus* laccase [2]. Dialysis against EDTA did not eliminate the low-field signal, indicating that the type 2 copper is tightly bound to a specific site, as in *Polyporus* laccase [12]. The estimated parameters (g_{II} and A_{II}) are 2.19 and 0.009 cm^{-1} for the narrowly spaced signal and 2.25 and ≥ 0.017 cm^{-1} for the widely spaced signal. These values fit well to values for type 1 and 2 coppers [2] and are closest to those of *Polyporus* (2.190 and 0.0090 cm^{-1}, 2.243 and 0.0194 cm^{-1}, respectively).

Laccase from *Polyporus versicolor* also contains two EPR-silent type 3 coppers [2]. According to the AAS results *Phlebia* laccase contains only two coppers. In keeping with this result, the absorption spectrum of concentrated *Phlebia* laccase does not contain the typical maximum of type 3 coppers at 330 nm [2], whereas the maximum of type 1 copper at 605 nm is clearly seen. Instead of type 3 coppers, a novel covalently bound cofactor PQQ was discovered in *Phlebia* laccase (Table I) using the hexanol extraction procedure [9]. PQQ was isolated as its 4-hydroxy-5-hexoxy-PQ derivative. The PQQ content per laccase molecule was 0.94 molecules as calculated from the molar absorption coefficient of the PQQ derivative, whereas a value of 0.90 was obtained by biological assay. The EPR spectrum of laccase (Fig. 1) did not include features characteristic of PQQ. Only the semiquinone form PQQH, created by a suitable electron donor, has an EPR signal [7]. Positioned at the $g = 2$ region this form, if present, would interfere with the copper. PQQ has been shown to function as a two-electron carrier when acting as a cofactor in oxidoreductases [7]. The type 3 coppers also accept electrons in pairs [2]. Recently it has been proposed that in blue oxidases a type 3 copper pair and one type 2 copper together represent a trinuclear copper site for oxygen binding and electron storage [13]. PQQ has been found in many mammalian and microbial oxidases in conjunction with type 2 copper(s) [7].

Table I

PQQ content of *Phlebia* laccase

Amount of enzyme (nmol)	PQQ detected (nmol)	
	by UV absorption	by biological assay
62.5	59.0	56.0
PQQ (mol per enzyme mol	0.94	0.90

Polyporus laccase catalyses the formation of phenoxy radicals from a phenolic compound by receiving one electron [1]. Monomeric radicals then react further nonenzymatically to yield quinones, polymers [1,14] or aromatic ring cleavage products [14]. During

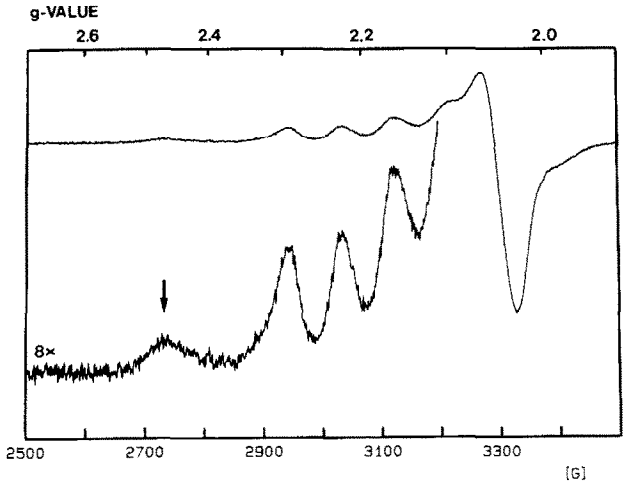

Fig. 1. $g = 2$ EPR spectrum of laccase from *Phlebia radiata*. The arrow points to the first hyperfine line of type 2 copper. Protein concentration 78 nmol/ml.

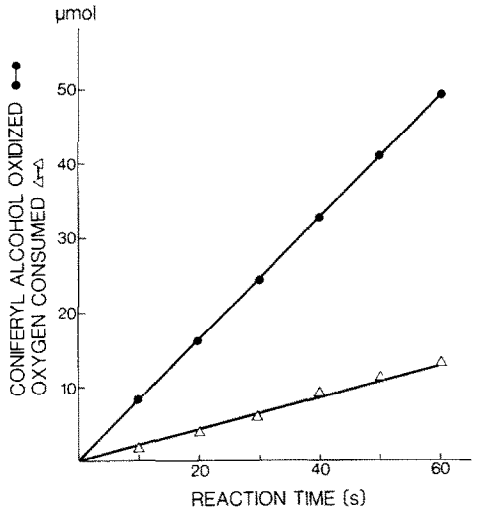

Fig. 2. Oxidation of coniferyl alcohol by *Phlebia* laccase. Coniferyl alcohol oxidized, (●—●); oxygen consumed, (△—△).

one catalytic cycle 4 electrons are transferred and molecular oxygen is reduced to H_2O. Stoichiometry and reaction mechanisms for the *Phlebia* laccase appear to be rather similar. Calculated from the simultaneous oxidation of coniferyl alcohol and oxygen consumption (Fig. 2), four molecules of coniferyl alcohol were oxidized per one molecule of oxygen. Because no oxygen production was observed on addition of catalase, the laccase obviously reduces O_2 directly to H_2O and not to H_2O_2. The reaction products detected by GLC-MS were the dilignols dehydrodiconiferyl alcohol, pinoresinol and an unidentified dimeric compound. These products have been proposed to be formed via quinone methides from coniferyl alcohol radicals [1].

The precise characterization of cofactors in fungal laccases has earlier been reported only for *Polyporus* [2] and *Neurospora* [15]. *Phlebia* laccase is the first example of a laccase containing PQQ. The laccases from *Agaricus* [3] and *Schizophyllum* [4], reported to contain two coppers but not characterized in detail, may in fact belong to the same category as *Phlebia* laccase.

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