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Excretion of laccase by sycamore (*Acer pseudoplatanus* L.) cambial cells: effect of copper deficiency, reversible removal of type 2 Cu^{2+}

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Introduction

Cambial cells of sycamore excrete a laccase-type polyphenol oxidase (EC 1.10.3.1) (Bligny and Douce, 1983). This type of enzyme, which utilizes molecular oxygen to oxidize phenolic substrates, is probably involved in lignin degradation processes (Mayer and Harel, 1979). This polyphenol oxidase is excreted by many mycorrhizal fungi (for a review, see Mayer, 1987) and is present in some higher plant cells including the genus *Rhus (Rhus vernicifera,* see Reinhammar, 1970), *Aesculus* (Wosilait *et al.*, 1954) and *Prunus* (Lehman *et al.*, 1974). The enzyme was purified from the nutrient medium, into which it was excreted by suspension-cultured sycamore cells, using classical purification techniques including a concanavalin A–Sepharose affinity column. The sycamore cell laccase is a monomeric blue copper protein containing 45% carbohydrate and 4 copper atoms (one type 1 and 2 Cu^{2+} and two type 3 Cu^{2+}) per molecule. The molecular mass (M_r = 97 000) was calculated from the sedimentation coefficient ($s_{20,W}$ = 6.1S), the diffusion coefficient ($D_{20,W}$ = 5.3), and the partial specific volume ($\bar{v}$ = 0.71). The specific activity of the purified enzyme measured at pH 6.6 (optimum pH) and in the presence of 20 mM 4-methylcatechol (optimum substrate conditions) corresponds to an oxygen uptake of at least 32 μmol of consumed O_2/min/mg of protein. The structure of the protein is stabilized by asparagine-linked oligosaccharides, which are a series of recurrent xylose-containing biantennary complexes that share at the core a common structural unit, *i.e.*, Xylβ 1 $\rightarrow$ 2(Manα 1$\rightarrow$6)Manβ 1 $\rightarrow$ 4GlcNAcβ 1 $\rightarrow$ 4(Fucα 1 $\rightarrow$ 3)GlcNAc (Takahashi *et al.*, 1986). The laccase excreted by the cells corresponded to *ca* 2% of the total protein synthesized during cell growth. The molecules are synthesized at the level of the endoplasmic reticulum where Cu atoms are probably incorporated and in the Golgi cisternae where the protein matures (glycosylation). The excretion process is inhibited by 1 μM monensin.

Effects of a copper deficiency

The total amount of active laccase excreted by sycamore cells was closely proportional to the amount of copper initially present in the culture medium (in the range of 2–100 μg of copper/l of nutrient medium, Bligny *et al.,* 1986). Copper-deprived cells excreted the apolaccase (laccase without copper, inactive) at the same rate as copper-supplied cells excrete the active laccase (hololaccase). The concentrated apolaccase (100 mg/ml) has a slightly yellow color contrasting with the deep blue color of hololaccase. As shown in Fig. 1, the absorption spectra of apolaccase showed a striking loss of absorption at 612 and 330 nm corresponding, respectively, to a strong decrease of type 1 and type 3 copper atoms. In addition, the EPR spectra (Fig. 2) show that the type 2 copper decreased in the same proportion. Addition of 2 μM copper to copper-deficient cultures triggers the excretion of hololaccase after a 5 h lag phase, corresponding to the time for

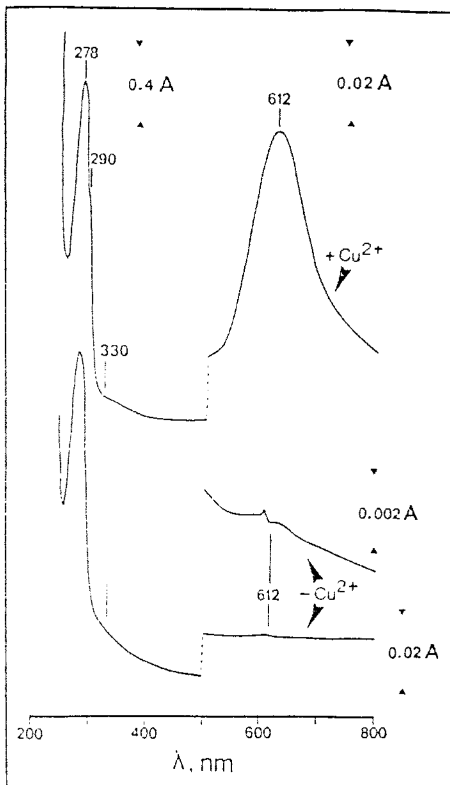

Fig. 1. Room-temperature absorption spectra of sycamore cell hololaccase ($+Cu^{2+}$) and apolaccase ($-Cu^{2+}$). Solutions containing 2.5 mg of laccase ($+Cu^{2+}$) or 2.5 mg of apolaccase ($-Cu^{2+}$) per ml were used. Optical path length, 10 mm (quartz cuvettes).

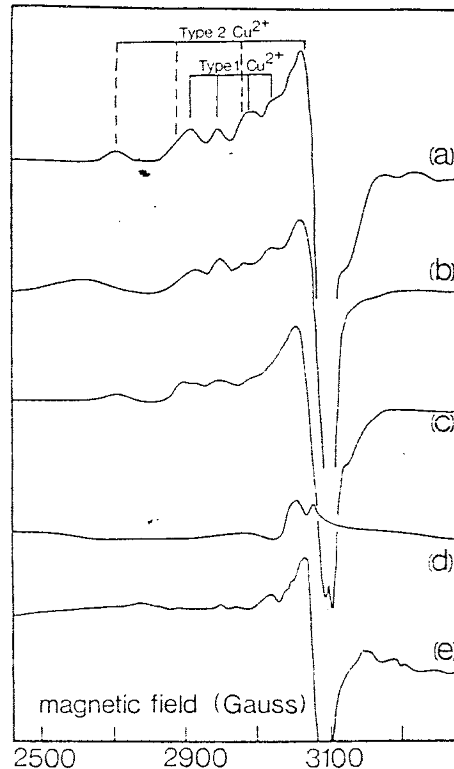

Fig. 2. EPR spectra at 40 K and 9.25 GHz of sycamore cell hololaccase and apolaccase subjected to different treatments. **a**: hololaccase (12 mg/ml); **b**: type 2 Cu^{2+}-depleted hololaccase (T2D-laccase, 17 mg/ml); **c**: reconstituted T2D-laccase (6 mg/ml); **d**: apolaccase (25 mg/ml); **e**: copper insertion assay in apolaccase (18 mg/ml).

maturation and excretion of the enzyme including the time necessary for incorporation of Cu into the catalytic center.

Preparation of type 2 Cu^{2+} (T2D) sycamore cell laccase; reconstitution assays

The type 2 copper atom was removed from the hololaccase according to the method of Morpurgo *et al.* (1980). 75 μM laccase was dialyzed for 12 h under anaerobiosis, against solution A, containing 2 mM dimethylglyoxime, 2 mM potassium ferrocyanide and 50 mM sodium acetate buffer, pH 5.2. After the first 8 h, 1 mM EDTA was added to solution A. The sample was then dialyzed anaerobically 3 times for 5 h against a rinsing solution containing 0.1 M phosphate buffer, pH 6.0.

After the first experiment, 50% of the type 2 Cu^{2+} was removed. Then the experiment was repeated and the type 2 Cu^{2+} was reduced to *ca* 20%. Optical and EPR spectra showed that the type 1 and 3 Cu^{2+} were not removed. Under these conditions, it was observed that the specific activity of this T2D-laccase was reduced to 6.5 μmol of O_2 consumed/min/mg protein, *i.e.*, to about 20% of the normal value. This indicates that the specific activity of sycamore cell laccase strictly depends upon the presence of the type 2 Cu^{2+}. This result was confirmed by reconstitution assays. The type 2 Cu^{2+} was reintroduced into the molecules of T2D-laccase in assays adapted from the method of Malkin *et al.* (1969). 25 μM T2D-laccase was anaerobically incubated for 1 h in solution B, containing 30 mM ascorbic acid, 50 μM copper sulfate and 10 mM sodium citrate, pH 6.0. The samples were then dialyzed for 20 h at 4°C against 2 changes of 25 mM potassium phosphate + 1 mM EDTA, pH 6.0, and concentrated by ultrafiltration (Diaflo XM50 membrane). As shown in Fig. 2, the type 2 Cu^{2+} atom was reincorporated into the T2D-laccase. Measurements of enzymic activity showed, therefore, that the specific activity of the reconstituted enzyme (35 μmol of O_2 consumed/min/mg protein) was fully recovered.

Copper introduction assays in sycamore cell apolaccase

In order to introduce the type 2 Cu^{2+} (and possibly the type 1 and 3 Cu^{2+}) into the copper-free laccase, we subjected the apolaccase to the same experiments as described above for the T2D-laccase. Surprisingly, it was not possible to introduce the type 2 Cu^{2+} into the copper-free laccase. The only modification observed on EPR spectra could correspond to copper atoms bound to the protein at non-specific sites. No enzymatic activity was detected in solutions of apolaccase subjected to copper introduction experiments.

In conclusion, since type 2 Cu^{2+} could be incorporated into T2D-laccase and not into apolaccase, it is possible that the presence of type 1 and 3 Cu^{2+} is necessary for the type 2 Cu^{2+} to be incorporated into the glycosylated apoprotein.

References

Bligny R. & Douce R. (1983) Excretion of laccase by sycamore (*Acer pseudoplatanus* L.) cells – purification and properties of the enzyme. *Biochem. J.* 209, 489-496

Bligny R., Gaillard J. & Douce R. (1986) Excretion of laccase by sycamore (*Acer pseudoplatanus* L.) cells – effects of a copper deficiency. *Biochem. J.* 237, 583-588

Lehman E., Harel E. & Mayer A.M. (1974) Copper content and other characteristics of purified peach laccase. *Phytochemistry* 13, 1713-1717

Malkin R., Malmstrom B.G. & Vanngard T. (1969) The reversible removal of one specific copper (II) from fungal laccase. *Eur. J. Biochem.* 7, 253-259

Mayer A.M. (1987) Polyphenol oxidases in plants – recent progress. *Phytochemistry* 26, 11-20

Mayer A.M. & Harel E. (1979) Polyphenol oxidases in plants. *Phytochemistry* 18, 193-215

Morpurgo L., Graziani M.T., Finazzi-Agrò A., Rotilio G. & Mondovi B. (1980) Optical properties of Japanese lacquer tree *(Rhus vernicifera)* laccase depleted of type 2 copper (II). *Biochem. J.* 187, 361-366

Reinhammar B. (1970) Purification and properties of laccase and stellacyanin from *Rhus vernicifera. Biochim. Biophys. Acta* 205, 35-47

Takahashi N., Hotta T., Ishihara H., Mori M., Bligny R., Akazawa T., Endo S. & Arata Y. (1986) Xylose-containing common structural unit in *N*-linked oligosaccharides of laccase from sycamore cells. *Biochemistry* 25, 388-395

Wosilait W., Nason A. & Terrell A.J. (1954) Pyridine nucleotide-quinone reductase II. *J. Biol. Chem.* 206, 271-282