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Excretion of laccase by sycamore (*Acer pseudoplatanus* L.) cells*

Effects of a copper deficiency

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Copper-deprived sycamore (*Acer pseudoplatanus*) cells do not excrete molecules of active laccase in their culture medium. In the range of 2–100 μg of copper initially present per litre of nutrient solution, the total laccase activity measured in the cell suspensions at the end of the exponential phase of growth was closely proportional to the amount of added copper. However, copper-deprived cells excreted the laccase apoprotein (laccase without copper) at the same rate as copper-supplied cells excreted the active, copper-containing, laccase. When the culture medium was initially supplied with limiting amounts of copper, the active laccase was excreted until all copper molecules were metabolized. Thereafter, the laccase apoprotein was excreted. Consequently, at the end of the exponential phase of growth, the cell supernatants contained a mixture of apoprotein and copper-containing laccase. After purification and concentration, this mixture of copper-containing laccase (blue) and laccase apoprotein (slightly yellow) showed a yellow–green colour. Under copper-limiting culture conditions an equivalent decrease of Type 1, Type 2 and Type 3 Cu^{2+} was observed. Addition of copper to copper-deficient enzyme solutions does not result in a recovery of the enzyme activity. However, when added to copper-deficient sycamore-cell suspensions, copper induced a recovery of the excretion of active enzyme, at a normal rate, within about 10 h. The first molecules of active laccase were excreted after 3–4 h.

INTRODUCTION

It has recently been demonstrated that suspension-cultured sycamore (*Acer pseudoplatanus*) cells excrete continuously a laccase-type polyphenol oxidase in their culture medium (Bligny & Douce, 1983). Like Japanese-lacquer-tree (*Rhus pernicifera*) laccase (Reinhammar, 1970), sycamore-cell laccase is a monomeric protein containing 45% carbohydrate and four copper atoms per molecule of M_r 97000. The specific activity of the purified extracellular sycamore-cell laccase 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 32 μmol of O_2/min per mg of protein (Bligny & Douce, 1983). Like cytochrome *c* oxidase, laccase can catalyse the reduction of dioxygen with the formation of 2 molecules of water. Both enzymes contain copper ions at their catalytic centre. As previously established (Bligny & Douce, 1977), a copper deficiency in sycamore-cell culture medium induced a dramatic decrease of spectrophotometrically detectable cytochrome *c* oxidase without modification of the respiration rates (since this enzyme is present in large excess in higher-plant cells). Thus we decided to verify whether a copper deficiency was also accompanied by a decrease of laccase activity in culture supernatants and whether the laccase apoprotein continued to be excreted under such conditions. On the other hand, it is now well established, in the case of *Rhus* laccase, that, of the four copper ions, two (Type 1 and Type 2 Cu^{2+}) are the primary acceptors of electrons from the reducing substrates (Reinhammar & Oda, 1979; O'Neill *et al.*, 1984) and are detectable by e.p.r. spectrometry, and two (Type 2 Cu^{2+}, antiferromagnetically coupled) are involved in the dioxygen-reducing site and are not e.p.r.-detectable (Malmström *et al.*, 1975). For many years, attempts have been made to modify the proportions of the three copper types in laccase, particularly by reversibly removing the Type 3 Cu^{2+} (Malkin *et al.*, 1969; Frank *et al.*, 1983; Morpurgo *et al.*, 1983). Thus we also decided to verify that copper-deficient culture conditions can modify the balance of copper ions in laccase.

EXPERIMENTAL PROCEDURES

Plant material

Sycamore (*Acer pseudoplatanus* L.) cells were cultivated in 20-litre batch cultures under previously described conditions (Bligny, 1977). The automatic recording of cell-suspension growth was carried out by means of turbidity measurements (Bligny, 1977),

The basic nutrient medium was prepared as described by Lamport (1964), as modified by Lescure (1966). In addition, 100 μg of copper were added per litre of standard culture medium in order to prevent any copper-deficiency effect. Copper-deprived medium contained less than 0.1 μg of copper/litre. The absence of fungal and bacterial contamination was verified in each culture.

* The present paper is part 2 of a series of papers on this topic; part 1 is Bligny & Douce (1983).

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Laccase activity measurements

Laccase activity of culture medium and purified laccase solutions was measured by means of a polarographic method. The reaction medium contained 50 mM-phosphate buffer, pH 6.7, and 20 mM-4-methylcatechol. O_2 uptake was monitored polarographically with a Clark-type oxygen-electrode system purchased from Hansatech, King's Lynn, Norfolk, U.K. The O_2 concentration in the air-saturated medium at 25 °C was taken as 240 μM.

Purification of laccase

The laccase excreted by sycamore cells in standard and copper-deficient culture medium was purified from 10-litre portions of culture as described previously (Bligny & Douce, 1983), with a modification for large volumes. After filtration on a glass-fibre filter, the culture supernatants were submitted to a first purification/concentration with an 8.0 cm × 20.0 cm DEAE-cellulose ion-exchange column (containing DE 32; Whatman, Maidstone, Kent, U.K.). The blue laccase, which was eluted with a solution containing 1% KCl and 10 mM-phosphate buffer, pH 6.5, was then further purified by using a concanavalin A–Sepharose column. When supernatants were taken from 10 litres of sycamore-cell suspensions at the growth plateau, the average yield of purified laccase was about 200 mg.

Concentrated enzyme solutions (50–100 mg of protein in 1 mM-phosphate-buffered solutions, pH 6.5) were deep blue in the case of standard laccase and yellow–green in the case of copper-deficient laccase. Stock solutions were stored desiccated below 0 °C.

Polyacrylamide-gel electrophoresis

Non-denaturing linear 7.5–15%-(w/v)-polyacrylamide-gel gradients (2 mm × 150 mm) were electrophoresed by the method of Davis (1964) at pH 8.9. Samples (50 μg) were subjected to electrophoresis for 5 h at 20 mA and 50 V. The protein bands were stained with 0.25% Coomassie Brilliant Blue in methanol/water/acetic acid (5:5:1, by vol.) for 4 h at 25 °C and destained in the same medium without the stain. In order to detect laccase activities on the gels, unstained gels were immersed in 10 mM-phosphate buffer containing 20 mM-catechol, at pH 6.8, for 1 h and rinsed in 10 mM-phosphate buffer for 1 h.

Immunochemical analyses

Purified laccase was injected into rabbits for raising antibodies. IgG fractions were purified from rabbit antisera by chromatography on DEAE-trisacryl M (IBF, Paris, France) by the method of Saint-Blancard *et al.* (1981). The immunochemical properties of the purified anti-laccase IgG were verified by double-immunodiffusion assays (Ouchterlony & Nilsson, 1973). Assays were performed overnight in agarose gel containing 1% (w/v) Triton X-100 as described by Chua *et al.* (1982).

Immunoblotting experiments were performed as described by Burnette (1981) after electrophoretic transfer of laccase from polyacrylamide-gel electrophoresis to nitrocellulose sheets by the method of Towbin *et al.* (1979). Incubation in the presence of IgG and labelling of the immunoprecipitates by ^{125}I-protein A was done as described by Block *et al.* (1983). Specific binding of antibodies to laccase was revealed by autoradiography of the nitrocellulose sheets with Kodak X-Omat X-ray films).

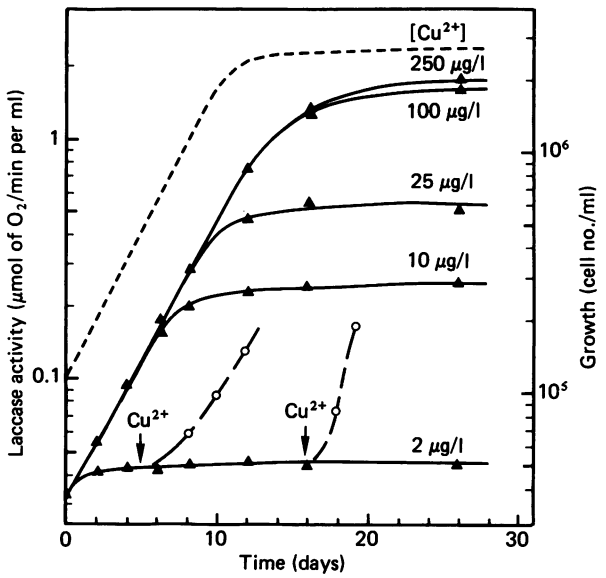

Fig. 1. Effect of the amount of copper initially added to nutrient medium on the total laccase activity (▲) measured in this medium during the growth of sycamore-cell suspensions

Cultures were grown as described previously (Bligny, 1977). Laccase activities were measured as described in the Experimental procedures section. The broken line without symbols indicates the cell-number growth [the cell-number growth was identical in the range of 2–250 μg of Cu^{2+} initially present per litre of nutrient medium (see Bligny & Douce, 1977)]. The arrows show the addition of copper (100 μg/litre) during the exponential phase of growth (left) or during the plateau (right).

E.p.r. spectrometry

E.p.r. spectra were taken at 9.25 GHz on a Varian E-109 spectrometer coupled to a Hewlett–Packard 9826 calculator. Low temperatures (40 K) were obtained with a liquid-helium transfer system (Oxford Instruments ESR 900). The temperature was monitored with a gold–iron/chromel thermocouple about 2 cm below the bottom of the e.p.r. tube in the flowing helium-gas stream. The magnetic field was calibrated by using a Varian 'gaussmeter'. Cu^{2+}–EDTA was used as standard.

General methods

Cell counting. This was performed under the microscope with the aid of a Nageotte cell. Before being counted, portions of culture were macerated in 10% (w/v) chromic acid as described by Butcher & Street (1960) and sonicated (20 s; 20 kHz; 80 W) with a Sonimass 250 T instrument (Ultrason, Annemasse, France).

Protein determination. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Control measurements were made by direct weighing after freeze-drying of portions of the enzyme. Thus it appeared that a good estimation of the protein content of sycamore-cell laccase solutions was given by multiplying by 1.2 the values obtained by the Lowry method.

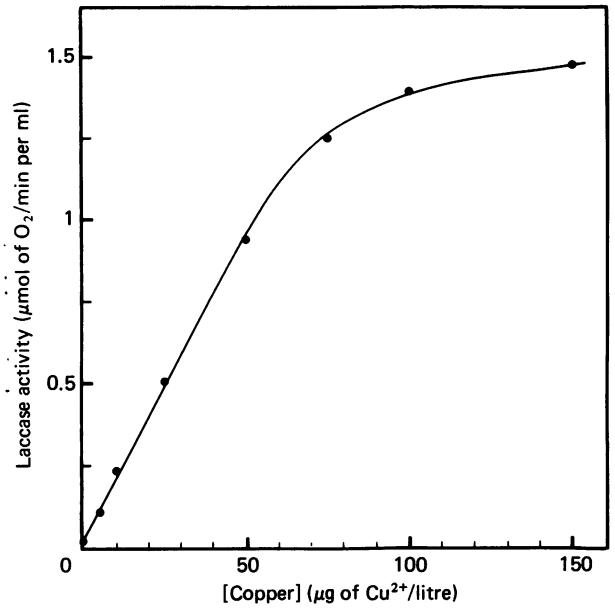

Fig. 2. Total laccase activity in sycamore-cell culture medium as a function of initial copper content of nutrient medium

Laccase activities were measured 1 week after the beginning of the plateau (see Fig. 1) as described in the Experimental procedures section.

Carbohydrate determination. The carbohydrate moiety of the enzyme was determined by the orcinol method (Vasseur, 1948).

Hydrolysis of carbohydrates. Purified enzyme was submitted to the action of glycosidases from *Charonia lampas* (Miles Laboratories, Elkhart, IN, U.S.A.) as follows. A 1 mg portion of purified laccase was incubated for 4 h at 37 °C in a solution containing 1 mg of glycosidases, 2 mM-phenylmethanesulphonyl fluoride and 50 mM-Mops buffer, pH 5.

M_r determinations. The M_r of purified proteins was calculated from the sedimentation and diffusion coefficients and from the partial specific volume as described previously (Bligny & Douce, 1983).

RESULTS

Effect of copper deficiency on the excretion of active laccase by sycamore cells

When sycamore cells are cultivated in a culture medium containing 100 μg of copper/litre (or more) at the beginning of the growth, the laccase activity in the supernatant increases exponentially during cell growth, reaching maximum values of 1300–1600 nmol of O_2 consumed/ml in the presence of 20 mM-4-methylcatechol, at pH 6.7 (Fig. 1). When the initial copper concentration in the culture medium was less than 100 μg/litre, the total maximum laccase activity present in the supernatant was proportional to the amount of available copper (Fig. 1 and Fig. 2). In copper-deprived cultures (less than 0.1 μg of Cu^{2+}/litre), sycamore cells do not excrete detectable amounts of active laccase. Interestingly, an initial concentration of copper of less than 2 μg/litre limited the maximum density of sycamore-cell cultures (Bligny & Douce, 1977). Such a result indicates that the initial concentration of copper required to prevent a limitation of the total growth of sycamore cells is much lower than that required to limit total laccase activity. Addition of copper to a copper-deficient culture restored the excretion of active laccase by the cells (Fig. 1).

Excretion of laccase apoprotein in copper-deficient cultures

Sycamore-cell laccase was purified from the supernatants of copper-deficient cultures (initially containing 4 μg of copper). The same purification techniques (Bligny & Douce, 1983) were used for normal and for copper-deficient cultures. Under these conditions, concentrated solutions of a yellow–green-coloured protein (50 mg/ml) were obtained, contrasting with the deep-blue colour of the normal laccase. The M_r of this protein determined from the diffusion coefficient ($D_{20,w} = 5.2$–5.3), the sedimentation coefficient ($s^0_{20,w} = 6.0$–6.1 S) and the partial specific volume ($\bar{v} = 0.71$) was 95000–100000 (limits of error obtained from six different experiments). This M_r corresponded to that of the normal laccase. Similarly, the carbohydrate coating of the molecule was not modified; the normal laccase and this protein contained 45% carbohydrate. Furthermore, this glycoprotein was excreted by sycamore cells at the same rate as normal laccase (3.5 μg/day per 10^6 cells). Consequently it was concluded that this protein was the laccase apoprotein. In addition, this copper-deficient laccase had a slight enzymic activity [2.5–2.7 μmol of O_2 consumed/min per mg of protein in

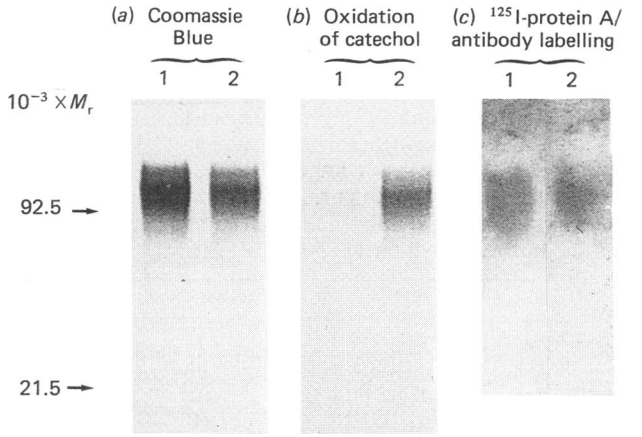

Fig. 3. Electrophoretic, enzymic and immunochemical characterization of normal (1) and copper-deficient (2) sycamore-cell laccase

Solutions containing respectively 20 and 30 μg of normal and copper-deficient laccase were submitted to polyacrylamide-gel electrophoresis with 7.5–15%-(w/v)-acrylamide gradients. The copper-deficient laccase was purified from copper-deficient cultures at the growth plateau (initial copper concentration of the nutrient medium: 4 μg of Cu^{2+}/litre). Gels were incubated (*a*) in presence of Coomassie Blue, (*b*) in a 20 mM-catechol solution for localization of enzymic activity, and (*c*) in presence of antibodies to laccase (see the Experimental procedures section) after electrophoretic transfer of polypeptides from polyacrylamide gels to nitrocellulose sheets. ^{125}I-protein A was used for the labelling of antigen–antibody complexes.

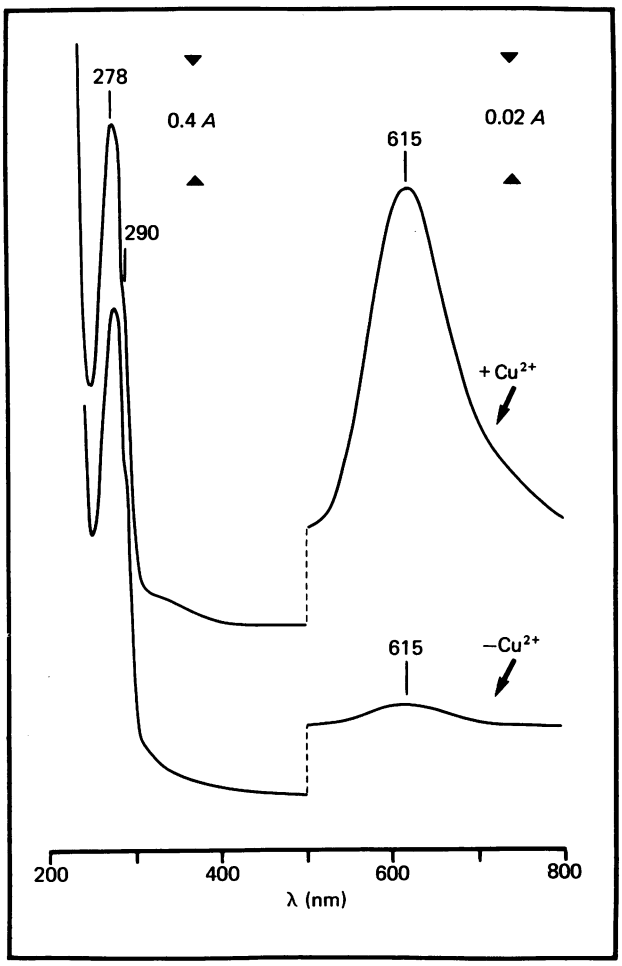

Fig. 4. Room-temperature absorption spectra of normal ($+Cu^{2+}$) and copper-deficient ($-Cu^{2+}$) sycamore-cell laccase

Solutions containing 2.5 mg of purified laccase/ml from normal and copper-deficient cells were used. The optical path length was 10 mm (quartz cuvettes). The copper-deficient laccase was purified from copper-deficient cultures at the growth plateau (initial copper concentration of the nutrient medium: 4 μg of Cu^{2+}/litre)

the presence of 20 mM-4-methylcatechol at pH 6.7 (32 μmol of O_2 for normal laccase)], corresponding to the residual presence of 0.018% copper (0.24% in normal laccase). These results are confirmed by the observation of electrophoretic profiles and antigenic reaction with antibodies to laccase (Fig. 3).

Recovery of the excretion of active laccase

After addition of copper (100 μg/litre) to copper-deficient cultures, the recovery of a normal excretion of active laccase by the cells took about 10 h (results not shown). During the first 2–3 h the excretion of active laccase was barely detectable. After this lag phase, the excretion of active enzyme increased progressively during 8–10 h and then reached the normal excretion rates observed with standard sycamore-cell suspensions. This excretion of laccase must correspond to the synthesis *de novo* of active enzyme, since it was not possible to activate the laccase apoprotein by addition of $CuSO_4$ (up to 1 mM), even under anaerobic reducing conditions (Malkin *et al.*, 1969).

Spectral properties of copper-deficient laccase

The absorption spectra of copper-deficient laccase at room temperature showed a striking loss of absorption at 330 nm and 615 nm (Fig. 4) corresponding respectively to a decrease in Type 3 Cu^{2+} and Type 1 Cu^{2+}. The decrease of Type 1 Cu^{2+} was closely proportional to the decrease of the total copper present in copper-deficient laccase (about 93% decrease). As Type 1 Cu^{2+} is

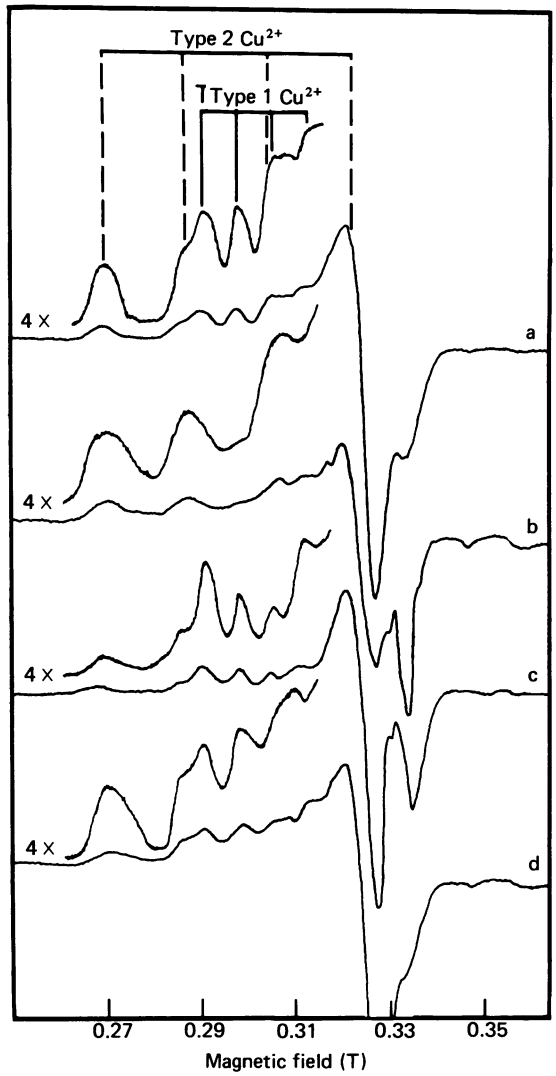

Fig. 5. E.p.r. spectra of native, copper-deficient and copper-depleted sycamore-cell laccase at 40 K and 9.25 GHz

a, Native laccase (10 mg/ml); *b*, native laccase (10 mg/ml) reduced anaerobically by 1 mM-4-methylcatechol; *c*, native laccase (10 mg/ml) treated with 5 mM-H_2O_2 for 15 s before freezing; *d*, copper-deficient laccase (50 mg/ml), i.e. laccase purified from copper-deficient sycamore-cell cultures (see the Experimental procedures section). Note that this sample contains five times more protein than native laccase. Conditions for e.p.r. spectra: microwave frequency 9.25 GHz; modulation amplitude 0.8 mT (8G); microwave power 2 mW; temperature, 40 K. All laccase samples were in 10 mM-phosphate buffer, pH 6.5.

Table 1. Composition and forms of copper in native and copper-deficient sycamore-cell laccase

Measurements were made by atomic absorption without flame for total copper, by e.p.r. spectrometry for Type 1 and Type 2 Cu^{2+} and by difference between total copper and Type 1+Type 2 Cu^{2+} for Type 3 Cu^{2+}. The copper-deficient laccase was purified from copper-deficient cultures at the growth plateau (initial copper concentration of the nutrient medium: 4 μg of Cu^{2+}/litre).

	Average number of ions per molecule			
Laccase species	Type 1 Cu^{2+}	Type 2 Cu^{2+}	Type 3 Cu^{2+}	Total Cu^{2+}
Native	1	1	2	4
Copper-deficient	0.08	0.08	0.16	0.32

responsible for the blue colour of the protein (absorption peak at 615 nm) and since the concentrated apoprotein devoid of copper is yellowish, this explains the slightly green colour of the copper-deficient laccase solutions. The e.p.r. spectrum of native sycamore-cell laccase at 9.25 GHz is given in Fig. 5, curve a. The Type 1 Cu^{2+} and Type 2 Cu^{2+} e.p.r. signals are specifically characterized in curves b and c of Fig. 5, where laccase was subjected respectively to 5 mM-H_2O_2 and to anaerobic reduction by 1 mM-4-methylcatechol. Type 3 Cu^{2+} is not detectable by e.p.r. spectrometry at any temperature between that of liquid helium and ambient. In the case of copper-deficient laccase (Fig. 5, curve d), the e.p.r. signals were diminished by 90%. However, no significant difference was observed in the balance of Type 1, Type 2 and Type 3 Cu^{2+} (Fig. 5 curve, d, and Table 1). Incubation of copper-deficient laccase with 1 mM-$CuSO_4$ under anaerobic reducing conditions did not result in the incorporation of copper atoms in either site. Finally, when the purified enzyme was submitted to the action of glycosidases from *Charonia lampas*, we observed a decrease in the different types of copper that was proportional to the laccase activity that was also observed.

DISCUSSION

The present results indicate that 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/litre of culture medium). Below 2 μg of Cu^{2+}/litre, the normal growth plateau (2.5–2.7 10^6 cells/ml) was not reached (Bligny & Douce, 1977); at over 100 μg of Cu^{2+}/litre, the limiting factor for protein synthesis became the nitrogen supply (Leguay & Guern, 1975). Between these two limiting values, normal laccase was synthesized until the copper concentration in the nutrient medium was very low (less than 1 μg of Cu^{2+}/litre). The synthesis and excretion of normal laccase was then replaced by the synthesis and excretion of copper-deficient laccase (apoprotein). This explains why, in the case of cultures grown in presence of limiting amounts of copper (i.e. 4 μg of Cu^{2+}/litre), the specific activity of laccase purified from the supernatant medium at the plateau was much lower than that of normal laccase. The decrease of specific activity paralleled the average decrease of copper per molecule of enzyme. The examination of e.p.r. spectra of normal and copper-deficient laccase showed that Types 1, 2 and 3 Cu^{2+} were equally diminished by copper deprivation, suggesting that the affinities of the three binding sites for copper in sycamore-cell laccase are similar.

These results also show that the synthesis of the apoprotein of sycamore-cell laccase (i.e. the polypeptidic moiety of the molecule synthesized at the level of the endoplasmic reticulum and selectively modified by glycosylation in the cisternae of the Golgi apparatus) was not controlled by copper ions. The transfer of laccase from the Golgi complex to the plasmalemma via secretory vesicles and the excretion process (exocytosis) were equally copper-independent, since the copper-deficient laccase (apoprotein) was excreted at the same rate as was the normal four-copper-ion-containing laccase. In addition, the time necessary for copper-deficient sycamore cells to excrete significant amounts of active laccase after addition of copper, i.e. 4–5 h, corresponds to the time of incorporation of copper atoms into the four catalytic sites of the apoprotein plus the time of glycosylation of the protein (maturation in Golgi cisternae) plus the time of transfer of laccase via the Golgi complex to the plasmalemma.

On the other hand, the fact that addition of copper to copper-deficient enzyme did not result in the incorporation of copper into either site indicates that, for the synthesis of normal active laccase, copper ions must be incorporated to the catalytic centres *before* the maturation of the enzyme in Golgi cisternae occurs, i.e. before the glycosylation of the apoprotein. This is confirmed reciprocally by treatment of laccase with glycosidases: this renders the copper atoms labile and impairs the enzymic activity. Thus it is likely that the carbohydrate coating (the sycamore-cell laccase is a glycoprotein that contains 45% carbohydrate) not only protects the catalytic centres of the enzyme and permit its excretion, but also prevents the access of Cu^{2+} to the binding sites.

Finally, the effects of a copper deficiency on the synthesis of active laccase by sycamore cells can be compared with its effects on the synthesis of spectrophorometrically detectable cytochrome oxidase (Bligny & Douce, 1977). In both cases, the synthesis of the normal copper-containing protein was closely proportional to the amount of copper ions available in the culture medium at low copper concentrations. However, in contrast with cytochrome oxidase, the synthesis of active laccase was not dependent on the cell growth: a good recovery of active laccase excretion can occur at the plateau when copper is added, whereas the appearance of spectrophotometrically detectable cytochrome aa_3 in

copper-deficient sycamore cells is a slow process that is governed by the synthesis of new mitochondrial material.

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