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

Purification and properties of the enzyme

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A laccase-type polyphenol oxidase is excreted by sycamore cells (*Acer pseudoplatanus* L.) cells. The enzyme has been purified by classical purification techniques. It is a blue copper protein of M_r 97 000, containing 45% carbohydrate and 0.24% copper. This protein consists of one single unit and the copper content corresponds to four copper atoms per protein molecule. 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) corresponded to an oxygen uptake of 32 000 nmol of O_2 /min per mg of protein. Under these conditions, the catalytic-centre activity of the enzyme reached 100 s^{-1} . The excretion of laccase by sycamore cells is significant, being about 2% of the total protein synthesized by the cells during the exponential phase of growth, and is independent of cell growth. The physiological significance and the problems raised by the passage of this protein across the cytoplasmic membrane are discussed.

Since the early work of Yoshida (1883), polyphenol oxidase activity in fungi and plants has been described by a number of workers [for a review, see Mayer & Harel (1979)]. Polyphenol oxidase activity is catalysed by two types of enzymes (all classified together as EC 1.14.18.1): (1) the catechol oxidase-type or *o*-diphenol: O_2 oxidoreductase, which is inhibited by CO (Keilin & Mann, 1939) and phenylhydrazine (Lerner *et al.*, 1971); (2) the laccase-type or *p*-diphenol: O_2 oxidoreductase, which is not inhibited by CO and phenylhydrazine. Both catechol oxidase and laccase are copper enzymes (Keilin & Mann, 1939; Kubowitz, 1938). However, whereas catechol oxidase is widely distributed in the Plant Kingdom and has been observed in tissue culture of various higher plants (Forrest, 1969; Stafford & Galston, 1970; Vernon & Straus, 1972; Volk *et al.*, 1977), laccase has been described mainly in fungi (for a review, see Levine, 1966) and rarely in plants. The main plants containing laccase include the genus *Rhus* (Japanese lacquer tree) (Yoshida, 1883; Nakamura & Ogura, 1966) and *Aesculus* (horse-chestnut tree) (Wosilait *et al.*, 1954), *Prunus persica* (peach) (Lehman *et al.*, 1974) and different species of the *Anacardiaceae* (a family that includes the mango fruit) (Joel *et al.*, 1978).

* The present paper is part 1 of a series of papers on this topic.

In the present paper we show that suspension-cultivated sycamore cells synthesize large amounts of a laccase-type polyphenol oxidase and that this enzyme, like the fungal laccase (Levine, 1966), is rapidly excreted in the culture medium.

Experimental

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) and as modified by Lescure (1966). Copper-deficiency effects on copper enzymes (cytochrome *aa_3*, polyphenol oxidase and superoxide dismutase) were prevented by addition of 100 μg of copper/litre to the culture medium (Bligny & Douce, 1977). The absence of fungal and bacterial contamination was verified in each culture.

Polyphenol oxidase activity measurements

The polarographic method was the most convenient for determining polyphenol oxidase activities (Mayer *et al.*, 1966). Unless otherwise mentioned,

the reaction medium contained 10mM-phosphate buffer, pH 6.6, and 20mM-4-methylcatechol. O_2 uptake was monitored polarographically with a Clark-type oxygen-electrode system purchased from Hansatech Ltd., King's Lynn, Norfolk, U.K. The O_2 concentration in the air-saturated medium at 25°C was taken as 240 μ M.

Purification of extracellular polyphenol oxidase

All steps were performed at 4°C. A typical preparation started with 500ml of sycamore cell suspension at the growth plateau. The cell suspension was first filtered on a glass-fibre filter and the remaining insoluble impurities present in the culture medium were eliminated by centrifugation (110000g) overnight. The clear culture medium obtained was then further purified by ultrafiltration (Amicon Diaflo ultrafiltration system model 420; XM-300 membrane) and concentrated to 20ml with an XM-100 membrane. The supernatant fluid was then applied to a concanavalin A-Sepharose column (1.0cm $\times$ 10.0cm; Pharmacia Fine Chemicals) at a flow rate of 10ml/h per cm². The column was washed with a medium containing 1mM- Ca^{2+} , 1mM- Mg^{2+} and 20mM-Tris/HCl, pH 7.4, until the A_{280} was negligible. As shown in Fig. 1, contaminant products were eliminated with the washing

medium. The polyphenol oxidase was eluted with a solution containing 1% (w/v) α -methyl manno-pyranoside, 1mM- $CaCl_2$, 1mM- $MgCl_2$ and 20mM-Tris/HCl, pH 7.4. The fractions containing the polyphenol oxidase activity were then pooled, dialysed against 10mM- KH_2PO_4 buffer, pH 7.4, and concentrated to 2ml with the Amicon Diaflo ultrafiltration system (model 12; XM 100 membrane). The enzyme was further concentrated by ultracentrifugation (300000g, 2h) in a Beckman Airfuge instrument. The translucent blue pellets were pooled; a solution containing approx. 10mg of polyphenol oxidase (40mg of protein/ml) was finally obtained. The average yield was 35–40%.

Polyacrylamide-gel electrophoresis

Non-denaturing 7.5% (w/v)-polyacrylamide gels (5mm $\times$ 120mm) were electrophoresed by the method of Davis (1964) at pH 8.9. Samples (100 μ g) were subjected to electrophoresis for 6h at 20mA and 50V. The protein bands were stained with 0.25% Coomassie Brilliant Blue in methanol/water/acetic acid (5:5:1, by vol.) for 4h at 25°C and destained in the same medium without the stain. In order to detect polyphenol oxidase activity, unstained gels were immersed in 20mM-catechol/

Fig. 1. *Concanavalin A-Sepharose chromatography of extracellular sycamore-cell polyphenol oxidase* Chromatography was performed as described in the Experimental section. The absorbance was measured and recorded with an ISCO UA-4 absorbance monitor; polyphenol oxidase activity (■) was measured in each fraction (2ml). The four fractions pooled (nos. 33–36 as indicated by the bar, ▮) corresponded to the recovery of 60% of the applied protein and 70% of the enzymic activity.

10 mM-phosphate buffer, pH 6.8, for 1 h and rinsed in 10 mM-phosphate buffer for 1 h. Thus it was verified that the brown band corresponding to the polyphenol oxidase activity accounted for at least 99% of the total protein applied to the gels. In addition, linear 7.5–15% polyacrylamide-gel gradients were performed to ascertain that no non-enzymic protein was coincident with the polyphenol oxidase band.

Denaturing gels (10% polyacrylamide/0.025% sodium dodecyl sulphate) were electrophoresed as described by Laemmli (1970).

The carbohydrate moiety of the polyphenol oxidase was detected on the gels with Schiff's reagent as described by Kapitany & Zebrowski (1973).

General methods

Cell counting was performed under the microscope with the help 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). 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 polyphenol oxidase solutions was given by multiplying by 1.2 the values obtained by the Lowry method.

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

Copper determination. Copper was measured by atomic absorption without flame (carbon rod) after formation of a complex with ammonium pyrrolidine dithiocarbamate and extraction by 4-methylpentan-2-one.

Molecular-weight determination. For the determination of the molecular weight of the purified enzyme, solutions containing 0.25, 0.5 and 1 mg of enzyme/ml in 1 mM-phosphate buffer, pH 7.5, were used and the following parameters were calculated: (1) the sedimentation coefficient $s_{20,w}^0$, measured by analytical ultracentrifugation at 50 000 rev./min with a MSE Centriscan 75 instrument equipped with schlieren optics; (2) the diffusion coefficient $D_{20,w}$, measured by a quasi-elastic light scattering by the method of Zulauf & Eicke (1979); (3) the partial specific volume of the enzyme, measured by using a DMA 601 M 2A PAAR densitometer.

Results

Excretion of polyphenol oxidase by sycamore cells

The addition of 20 mM-4-methylcatechol to sycamore cell cultures in which cell respiration had

been blocked by antimycin A induced a rapid increase of O_2 uptake (Fig. 2a). When the cells were washed with fresh nutrient medium, the O_2 -uptake was much lower (Fig. 2b). The same low polyphenol oxidase activity was observed with washed cells disrupted by sonication (Fig. 2c). In contrast, the cell-free supernatant oxidized the phenolic substrate very rapidly (Fig. 2d). Consequently these results demonstrate that a polyphenol oxidase-type enzyme was excreted by sycamore cells.

In addition, Fig. 3 shows that the polyphenol oxidase activity was proportional to the cell number up to 2.10^6 cells/ml (i.e. during the exponential phase of growth), indicating that the production of polyphenol oxidase by sycamore cells was constant with time. The average excretion of enzyme per day corresponded to an increase of 90–100 nmol of O_2 uptake/min per 10^6 cells. At the end of the exponential phase of growth, the rate of enzyme excretion remained unchanged; it decreased significantly only 4 or 5 days later. At that time the polyphenol oxidase activity of sycamore cell suspensions reached 1300–1600 nmol of O_2 consumed per ml in presence of 20 mM-4-methylcatechol, at pH 6.6. Using the same procedure, we observed that polyphenol oxidase was excreted at a normal rate by non-growing sycamore cells [cells maintained in a culture medium deficient in the growth hormone 2,4-dichlorophenoxyacetic acid ('2,4D')]. Consequently, the excretion of polyphenol oxidase by sycamore cells is independent of cell growth.

Properties of the polyphenol oxidase of sycamore cells

Substrate specificity and sensitivity to inhibitors. Different phenolic substrates were tested (Table 1). It is clear that both *o*- and *p*-diphenol-type substrates, including the *o*-diphenols of the catechol type, were oxidized. In contrast, the sycamore polyphenol oxidase was unable to oxidize *p*-cresol and tyrosine. These properties are characteristic of higher-plant laccases (Mayer & Harel, 1979). On the other hand, the enzymic activity was decreased by 80% in the presence of 1 mM-KCN (Fig. 4) and by more than 60% in presence of copper-complexing agents such as 10 mM-EDTA (results not shown). However, CO and phenylhydrazine had no inhibiting effect (phenylhydrazine was oxidized by sycamore polyphenol oxidase). Consequently it is clear that the polyphenol oxidase excreted by sycamore cells is a laccase-type enzyme (Keilin & Mann, 1939; Lerner *et al.*, 1971).

Finally, this enzyme was inhibited by 50–60% in the presence of 2 mM-propyl gallate (2,4,5-trihydroxybenzoic acid propyl ester).

Specific activity. In the presence of 4-methylcatechol, the oxidation reaction catalysed by the sycamore-cell laccase had an apparent K_m of 20 μ M

Fig. 2. Polyphenol oxidase activity in sycamore-cell cultures

Cultures were grown as described previously (Bligny, 1977). Portions of the culture were taken at the end of the exponential phase of growth. (a) Cells in their growth medium. The cell concentration was 2×10^6 cells/ml. (b) Cells in fresh medium. Cells were washed twice with fresh medium, strained on a glass-fibre filter [15 s; pressure of suction 20 kPa (0.2 bar)]. The cell concentration was 2×10^6 cells/ml. (c) Cellular extract. Washed concentrated cells (4×10^6 cells/ml, i.e. 110 mg of cell fresh wt./ml) were disrupted by sonication [3 min, 20 kHz, 120 W, with a Sonimass 250 T instrument (Ultrason, Annemasse, France)] and cell walls were eliminated by centrifugation (1000 g). (d) Growth medium where sycamore cells were cultivated until a cell concentration of 2×10^6 cells/ml was reached. Polyphenol oxidase activities were measured as described in the Experimental section. Italic numbers on traces refer to nmol of O_2 consumed/min per ml. Given concentrations are final concentrations. Abbreviation used: 4-MC, 4-methylcatechol.

Fig. 3. Polyphenol oxidase activity in sycamore-cell-culture medium during the cell suspension growth. Polyphenol oxidase activities were measured as described in the Experimental section.

for O_2 and 4.5 mM for the phenolic substrate (Fig. 5) and exhibited a rather broad optimum pH between pH 6.6 and 6.8 (Fig. 6). Under these conditions, the specific activity of the purified extracellular sycamore-cell laccase measured at pH 6.6 in the presence of 20 mM-4-methylcatechol corresponded to an oxygen uptake of 32 000 nmol of O_2 /min per mg of protein or to a substrate oxidation of 64 000 nmol of 4-methylcatechol/min per mg of protein.

Stability. At room temperature and in a pH 7.5 phosphate-buffered solution, the enzyme activity decreased very slowly (1–2%/day). At 4°C and at pH 7 the sycamore-cell laccase can be conserved for several days without significant loss of activity, and for months in the frozen state. However, we noticed irreversible losses of activity at 4°C below pH 4 and above pH 10. Thus the extracellular sycamore-cell laccase is a remarkably stable enzyme.

Absorption spectrum and copper content. In the visible region the laccase spectrum contained an

Table 1. Substrate specificity of sycamore-cell polyphenol oxidase

Measurements were made at pH 6.6 in the presence of 20 mM-substrate, as described in the Experimental section.

Substrate	Polyphenol oxidase activity (nmol of O ₂ consumed/min per mg of enzyme)
4-Methylcatechol	32 000
Catechol	8 700
<i>p</i> -Cresol	(Trace)
Tyrosine	0
Pyrogallol	32 000
Guaiacol	190
α -Naphthol	8 000
Phenylhydrazine	2 300
Hydroquinone	26 000
Vanillin	0
Caffeic acid	0
Ferulic acid	0
Resorcylic acid	0
Coumarin	0
Chalcone	28 000
Phloroglucinol	500
Quercetagetin	1 100
Quercetin	20 000
Apigenin	0
Luteolin	3 800
Rhamnetin	5 000
Kaempferol	0

absorption peak at about 612 nm (Fig. 7). This is the origin of the deep-blue colour of concentrated enzyme solutions. In the u.v. region the spectrum contained an absorption peak at 278 nm (with a

Fig. 5. Lineweaver-Burk plots of initial O₂-uptake rates catalysed by sycamore-cell polyphenol oxidase as function of (a) 4-methylcatechol concentration in the presence of excess O₂, and (b) O₂ concentration in the presence of excess 4-methylcatechol

Enzymic activities were measured as described in the Experimental section.

Fig. 4. Effect of cyanide on sycamore-cell polyphenol oxidase activity

Solutions containing 13 μ g of purified polyphenol oxidase were used. Enzymic activities were measured as described in the Experimental section. Italic numbers on traces refer to nmol of O₂ consumed/min per ml. Given concentrations are final concentrations. Abbreviation used: 4-MC, 4-methylcatechol.

shoulder at 290nm), typical for a protein, and a slight shoulder at about 330–340nm. According to Reinhammar (1979) the absorption peaks at 612nm and 330–340nm belong respectively to Type 1 Cu(II) and to the Type 3 Cu(II).

The absorption coefficient at 280nm for a 1%

purified sycamore-cell laccase solution ($A_{280}^{1\%}$) was 8.2.

The copper content of the sycamore-cell laccase, measured as described in the Experimental section, was $2.4\mu\text{g}$ of Cu/mg of enzyme protein.

Carbohydrate content. The carbohydrate moiety of the sycamore-cell laccase measured by the orcinol method, with mannose as standard, was approx. 45% by weight.

Molecular weight of the extracellular sycamore-cell laccase. The sedimentation coefficient ($s_{20,w}^0$) of the sycamore-cell laccase was found to be 6.1S; the diffusion coefficient ($D_{20,w}$) 5.3, the partial specific volume ($\bar{v}$) 0.71. Consequently the molecular weight of the extracellular sycamore-cell laccase calculated from these data is 97000. This value was obtained from six different experiments and the limits of error were 92000–102000. Electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulphate indicated that the enzyme contains one single unit.

Discussion

These observations demonstrate that polyphenol oxidase is excreted by suspension-cultured sycamore cells. As the activity of the excreted polyphenol oxidase is not inhibited by CO nor by phenylhydrazine, and from the substrate specificity, it was concluded by Mayer & Harel (1979) that it is a laccase-type enzyme. The higher-plant laccases, as far as is known, are intracellular and localized in the cytoplasm as soluble enzymes. In contrast, fungal laccases are readily excreted by the

Fig. 6. Effect of pH on sycamore-cell polyphenol oxidase activity

Solutions containing $10\mu\text{g}$ of purified polyphenol oxidase per ml were used. Enzymic activities were measured as described in the Experimental section. The broken line shows O_2 uptake by autooxidation of 4-methylcatechol at alkaline pH.

Fig. 7. Room-temperature absorption spectrum of sycamore-cell polyphenol oxidase

A solution containing 1.1 mg of purified polyphenol oxidase/ml was used. The optical path-length was 10 mm (quartz cuvettes).

hyphae (Levine, 1966) The present results established that, in sycamore cells, the laccase-type polyphenol oxidase is an extracellular enzyme. Consequently it is possible that in higher-plant tissues the presence of laccase reported by several authors as a soluble form in the cytoplasmic fraction could represent an artefact arising during the grinding of the tissue. As a matter of fact it is difficult to distinguish a truly cytoplasmic enzyme from an enzyme embedded in the cell wall. Once the tissue is pulverized, there is the possibility of substantial contamination of cytoplasmic fractions and vacuolar system by external enzymes.

The sycamore-cell laccase is a blue glycoprotein that contains 45% carbohydrate, has mol.wt. 97000 and consists of one unit. The copper content of this laccase is 0.24% and consequently corresponds to an average of 3.7 copper atoms per protein molecule. As small amounts of copper could be lost during the enzyme preparation, it is assumed that sycamore-cell laccase typically contains four copper atoms per molecule. Thus the properties of sycamore-cell laccase are more similar to those of *Rhus* laccase (Reinhammar, 1970), which contains 45% carbohydrate and four copper atoms per protein molecule of mol.wt. 110000, than to those of peach (*Prunus persica*) laccase (Lehman *et al.*, 1974), which contains 25% carbohydrate and two copper atoms per protein molecule of mol.wt. 73500. It also differs significantly from fungal laccase (Froehner & Erikson, 1974). In addition, the catalytic-centre activity of the sycamore-cell laccase with 4-methylcatechol as substrate, calculated from the molecular weight (97000) and from the specific activity of the enzyme (64000nmol of substrate oxidized/min per mg of protein), has the same value, about 100s^{-1} , as that reported for *Rhus* (lacquer-tree) laccase by Reinhammar (1970).

The excretion of laccase by sycamore cells is significant; from the data of Fig. 3, and since 10^6 cells contain 0.25–0.30mg of protein, one can calculate that, during the exponential phase of growth, the synthesis of laccase by sycamore cells accounts for about 2% of the total synthesized proteins. What is the physiological significance of the huge excretion of a laccase-type polyphenol oxidase by sycamore cells? It is well established that laccase is involved in the oxidation of lignin by fungi (Ander & Eriksson, 1976), and it can be supposed that this enzyme could play an important role in the synthesis and deposition of specific wall substances such as lignin. In addition we have observed that sycamore cambial tissues sampled directly on trees contained non-negligible amounts of laccase mainly in the area where cambial cells differentiate into wood vessels (results not shown). However, no direct evidence of a role of laccase in lignin synthesis has been given, and it is clear that a great

deal of research is needed in order to understand the true function of the extracellular sycamore-cell laccase.

Finally, the results obtained in the present study raise the problem of the passage of a ~ 100000 Da protein across the cytoplasmic membrane. It is possible that this enzyme is synthesized in the rough endoplasmic reticulum, extruded into its cisternal space and then transferred via the Golgi complex to secretory vacuoles before being eliminated from the cell (Palade, 1975). It is also possible that it is synthesized on free ribosomes and its passage across the plasma membrane is associated with a proteolytic cleavage (see Chua & Schmidt, 1979).

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