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Some Enzymes Present in the Walls of Mesophyll Cells of Tobacco Leaves

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Cell-wall enzymes were assayed by the difference between enzyme activities in the whole cell and the protoplast. Both peroxidase (85.2%) and acid phosphatase (21.9%) were located in the wall. However, malate dehydrogenase was found only in the protoplast. A study of the time-course of the release of peroxidase and malate dehydrogenase into the incubation medium from cells either treated with cellulase or untreated, also indicated that peroxidase and not malate dehydrogenase was located in the wall. Only two anodic isoenzymes of peroxidase were present in the cell wall. These were more negatively charged than those of horseradish peroxidase.

The plant cell wall is not only a physical support and barrier, but a biochemical and physiological entity of the cell. Many enzymes have been reported in the wall (Lamport & Northcote, 1960; Kivilaan *et al.*, 1961; Chang & Bandurski, 1964; De Jong, 1967; Ridge & Osborne, 1971; Stephens & Wood, 1974). However, it is difficult to assess whether the enzymes found in isolated walls are artifacts from contamination by other cell fractions.

Tobacco mesophyll cells are used extensively in the study of protoplast culture and Nagata & Takebe (1971) have reported the successful regeneration of these protoplasts into callus tissue. In the present investigation, we report the distribution of peroxidase and acid phosphatase in the wall of tobacco mesophyll cells and also describe an alternative technique for the study of the enzymes that are present in the cell wall.

Materials and Methods

Microscopy

The cell and protoplast preparations were examined by bright field and Normarski differential interference optics on a Zeiss Ultraphot instrument for the cleanliness of the preparations. The preparations were stained with Calcoflour (white M2RS; American Cyanamide Co., Bound Brook, N.J., U.S.A.) and examined for fluorescence with u.v. optics for the presence of the cellulose (Bright & Northcote, 1974).

Spectrophotometry

All spectrophotometric measurements were carried out by a double-beam spectrophotometer (Beckman DB GT).

Enzymes

Horseradish peroxidase was purchased from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. Macerozyme (pectinase) and Onozuka cellulase, products of All Japan Biochemicals Co., were purchased through R. W. Unwin and Co., Welwyn, Herts., U.K.

Cell preparation

Mature leaves from tobacco plants (*Nicotiana tabacum* cv. Xanthi, 9–15 weeks old, grown in a greenhouse) were washed in water (1 min), Milton solution (Richardson–Merell Ltd., London, U.K.) (1 min) and 95% (v/v) ethanol (0.5 min) and then rinsed at least five times with 800 ml batches of water. After the edges, midribs and large veins were removed, the leaves were cut into fine strips and incubated (27°C; 2–4h; 10g of leaf tissue, 70ml of solution) in 2.5% (w/v) Macerozyme (pectinase) solution containing 0.7M-sorbitol and 2% (w/v) sucrose either in water or in the medium described by Nagata & Takebe (1971).

At the end of the incubation, the medium was filtered through muslin to separate the undigested tissues from the isolated cells. The filtrates were left to settle, and the supernatant containing chloroplasts and broken cells was removed with a Pasteur pipette. The concentrated cell preparation was then resuspended in 50ml of the above medium, and the supernatant was removed in the same manner. This process was repeated at least five times until the preparation examined under the microscope appeared clean.

Protoplast preparation

The concentrated cell preparation (10ml) was treated with Onozuka cellulase (2.0–2.5%, w/v) that had been purified by passage through a column (5.5cm×40cm) of Sephadex G-25 (Hanke & Northcote, 1974). After incubation at 27°C for 2–4h, the medium was passed through a stainless-steel screen (61 μm) to remove the undigested cells. The suspension

of protoplasts was left to settle and the supernatant removed. The protoplasts were washed five or six times with the medium until the preparation examined under the microscope showed no free chloroplasts or cell-wall fragments. It was stained with Calcoflour and showed no fluorescence when examined with u.v. optics, which indicated that no cell walls were present in the preparation.

Chlorophyll determination

The cell or protoplast preparation (0.2ml) was extracted with 80% (v/v) acetone in a 10ml flask. The solution was filtered and the absorbance measured at 652nm by the method of Arnon (1949).

Enzyme extraction

The cell or protoplast preparations were homogenized in the incubation medium in a pre-chilled mortar with acid-washed sand. The homogenates were then centrifuged by a Sorvall centrifuge at 38000*g* for 20min. The supernatants, free from cell organelles, were stored at 0°C.

Enzyme assays

Peroxidase activity. The total enzyme activity was assayed by the method of Chance & Maehly (1955). The reaction mixture contained 1ml of sodium phosphate buffer (10mM, pH7.0), 2ml of guaiacol (20mM) and 0.1ml of enzyme extract. The reaction was started by the addition of 0.1ml of H_2O_2 [20mM, freshly diluted from 30% (w/v) H_2O_2]. The change in E_{470} of the mixture was measured over 3min. One unit of peroxidase activity was defined as that which brings about an increase of 0.01 in the E_{470}/min.

Acid phosphatase activity. The acid phosphatase activity was determined by the modified method of Igarashi & Hollander (1968). The assay mixture (1ml) contained 0.5ml of sodium acetate (0.2M, pH5.0), 0.3ml of *p*-nitrophenyl phosphate (27.74mM) and 0.1ml of enzyme extract. The remaining volume was made up with water. After incubation at 37°C for 10min, the reaction was stopped by addition of 1.5ml of NaOH (0.25M). The liberated *p*-nitrophenol was measured by reading the E_{410}. One unit of enzyme activity was defined as that which brings about an increase of 0.01 in the E_{410}/10min.

Malate dehydrogenase activity. The malate dehydrogenase activity was determined by the method described in the *Worthington Enzyme Manual* (1972). The assay mixture (3ml) contained 2.6ml of sodium phosphate buffer (0.1M, pH7.4), 0.2ml of NADH (3.9mM), 0.1ml of oxaloacetate (8.2mM) and 0.1ml of enzyme extract. Both NADH and oxaloacetate were dissolved in 0.1M-phosphate buffer, pH7.4. The change in E_{340} of the mixture was measured over 7min. One unit of malate dehydrogenase was defined as that which brings about the change of 0.01 in the E_{340}/min.

Polyacrylamide-gel electrophoresis

The enzyme extracts were separated into isoenzymes by polyacrylamide-gel electrophoresis on the basis of the method of Davis (1964). The gel was composed of 1 part of solution A (Tris, 36.6g; 1M-HCl, 48ml; *NNN'N'*-tetramethylenediamine, 0.23ml; made up to 100ml with water, pH8.9), 2 parts of solution B (acrylamide, 28.0g; bisacrylamide, 0.735g), 1 part of water and 4 parts of freshly prepared ammonium persulphate solution (1.4mg/ml). The electrophoresis was run for 2.5h at 2.5mA per column (7cm×0.5cm). The gels were stained for peroxidase activity.

Results and Discussion

Enzyme activities in incubation medium

Equal concentrations of isolated cells were incubated in media either with or without cellulase, and enzyme activities in the media were assayed at different time-intervals (Fig. 1). With those incubations containing cellulase a steady increase of peroxidase activity was observed for 3.5h. There was no peroxidase present in the medium in the absence of cellulase. No malate dehydrogenase activity was found in the incubation medium whether cellulase was present or not.

It appeared therefore that when the polysaccharide molecules of the wall were degraded by the action of cellulase, peroxidase was released into the medium.

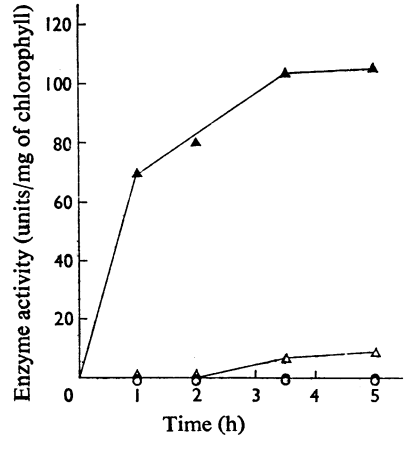

Fig. 1. *Peroxidase released from mesophyll cells in the presence* (▲) *and absence* (△) *of cellulase, and malate dehydrogenase released in the presence* (●) *and absence* (○) *of cellulase*

The enzyme activities were measured in samples of clear supernatants obtained by centrifugation of the incubation mixture. The assay procedures are described in the Materials and Methods section.

Table 1. *Peroxidase and malate dehydrogenase activities in cells, protoplasts and cell walls*

Chlorophyll concentration in both cell preparation and protoplast preparation was 0.17 mg/ml. The cells were isolated from the leaf by incubating the tissue with Macerozyme in 0.7 M-sorbitol and 2% (w/v) sucrose and protoplasts were prepared from the isolated cells by treatment with cellulase.

Fraction	Peroxidase (units/mg of chlorophyll)	Peroxidase (%)	Malate dehydrogenase (units/mg of chlorophyll)
Whole cell	220	100	30.6
Protoplast	32.6	14.8	29.9
Cell wall (whole cell −protoplast)	187.4	85.2	—

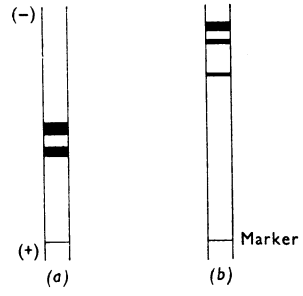

Fig. 2. *Isoenzymes of peroxidase*

(*a*) Whole-cell extract: concentration of enzyme applied was equivalent to 0.04 mg of chlorophyll. (*b*) Horseradish peroxidase: 6 μg was used. The isoenzymes were separated by polyacrylamide-gel electrophoresis for 2.5 h at 2.5 mA per column. The gels were stained with H_2O_2 and guaiacol.

Table 2. *Acid phosphatase in cells, protoplasts and cell walls*

Chlorophyll concentration in cell preparation was 0.30 mg/ml and in protoplast preparation 0.58 mg/ml. The cells were isolated from the leaf by incubating the tissue with Macerozyme in 0.7 M-sorbitol and 2% sucrose and protoplasts were prepared from the isolated cells by treatment with cellulose.

Fraction	Acid phosphatase (units/mg of chlorophyll)	Acid phosphatase (%)
Whole cell	333	100
Protoplast	258	78.1
Cell wall (whole cell −protoplast)	75	21.9

The failure to detect any malate dehydrogenase in the medium suggested that this enzyme was not located in the wall.

Peroxidase in protoplast and cell

The peroxidase activities in the protoplasts and the cells are shown in Table 1. The enzyme activity in the cells was approximately 8 times as high as in the protoplasts. The enzyme in the cell wall was calculated from the difference in enzyme activity between the cells and the protoplasts. Approx. 85.2% of the total peroxidase was located in the wall.

Malate dehydrogenase in protoplast and cell

In contrast with the report by Stephens & Wood (1974), malate dehydrogenase activity was the same in the cell (30.6 units/mg of chlorophyll) and in the protoplasts (29.9 units/mg of chlorophyll). Our data indicated that malate dehydrogenase is only located in the protoplast and not in the cell wall. As a result, malate dehydrogenase activity was used to serve as an internal standard and reference for the cleanliness and integrity of the cell and protoplast preparations.

Polyacrylamide-gel electrophoresis

Fig. 2 shows the isoenzymes of peroxidase in the cell extracts and in horseradish peroxidase. No anodic isoenzymes could be detected in the protoplasts, although the concentration of the chlorophyll equivalent to the extract applied to the column was five times higher than that of the cell extract. Two isoenzyme bands of peroxidase were present in the whole cell and three in the horseradish peroxidase. The isoenzymes of peroxidase in the walls of the tobacco mesophyll cells were more negatively charged than those of the horseradish peroxidase.

Thus two anodic isoenzymes of peroxidase were entirely located in the cell wall of the mesophyll cells of tobacco leaves and these were not present in the protoplasts. These results agree with the recent report by Mader *et al.* (1975), who found that two groups of anodic isoenzymes of peroxidase were present in the walls of cells of various tobacco tissues.

Test of peroxidase and acid phosphatase activity in Macerozyme and Onozuka cellulase

No peroxidase activity could be detected in the Macerozyme and Onozuka cellulase. The addition of Macerozyme or cellulase to horseradish peroxidase neither stimulated nor inhibited the activity of peroxidase activity.

No acid phosphatase activity was found in the Macerozyme. However, acid phosphatase activity was found in the Onozuka cellulase (187.5 units/mg dry wt. of Onozuka cellulase).

Acid phosphatase in protoplast and cell

In the determination of acid phosphatase activity in the protoplast, the presence of acid phosphatase in Onozuka cellulase was taken into account. The acid phosphatase remaining in a definite volume of the medium from the final wash in the protoplast preparation was measured and this value was subtracted from the amount determined for an appropriate volume of the protoplast suspension.

The activity of acid phosphatase was higher in the cell extract than in the protoplast extract (Table 2). The difference in acid photophatase activity was assumed to be due to the presence of cell walls. Approx. 22% of the acid phosphatase was located in the wall.

Presence of enzymes in the cell wall

Jansen *et al.* (1960) reported that there is a high binding affinity between cell walls and protein, so that enzymes located in isolated cell-wall fractions could result from contamination by other cell fractions or by binding of the enzymes to the walls during homogenization.

In the present investigation, the wall enzymes were assayed by the difference between the activities of the enzymes in the cell and those in the protoplast. This method eliminates the artifacts resulting from contamination by other cell fractions.

Peroxidase has been reported in many plant tissues. The presence of this enzyme in different cell fractions varies with the isolation procedures, but most studies indicate a localization in the cell wall (De Jong, 1967; Mader *et al.*, 1975; Ridge & Osborne, 1971). In the present study, almost all the peroxidase was located in the cell wall of the mesophyll cell of tobacco.

Plant growth hormones such as indol-3-ylacetic acid, cytokinin and ethylene can induce the synthesis of peroxidase (Galston *et al.*, 1968; Gayler & Glaszious, 1969; Ridge & Osborne, 1971; Lee, 1974). It has been suggested that peroxidase plays an important role in lignin formation (Harkin & Obst, 1973), metabolism of aromatic compounds (Stafford, 1974), auxin oxidation (Hinman & Lang, 1965) and ethylene production (Yang, 1967). The presence of peroxidase in the wall might therefore be a significant factor in the growth and differentiation of the cell.

Acid phosphatases have been reported in many plant tissues (Joyce & Grisolia, 1960; Newmark & Wenger, 1960; Forti *et al.*, 1962; Shaw, 1966), especially in ripening fruits (De Leo & Sacher, 1970; Rhodes & Wooltorton, 1967). The presence of this enzyme in the cell wall of callus tissue has also been reported (Lamport & Northcote, 1960).

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