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Short Communication

Soluble Peroxidase in Fluid from the Intercellular Spaces of Tobacco Leaves[1]

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ABSTRACT

A high proportion of the soluble peroxidase of tobacco (*Nicotiana tabacum* L. var. Bottom Special) leaves is found in the fluid obtained by centrifugation of a buffer solution previously infiltrated into the intercellular spaces. Only a very small amount of the cytoplasmic enzyme, glucose 6-phosphate dehydrogenase, is present in this fluid. Specific activity data suggest that an active process is responsible for the transfer of soluble peroxidase to the intercellular space and that the intercellular fluid fraction is not simply composed of material moving out of leaf cells by diffusion. The centrifugation method is a satisfactory means of isolating diluted intercellular fluid for biochemical and physiological investigations.

Klement (3) described a method for the isolation of a fluid from the intercellular spaces of tobacco leaves. In this method, the spaces were first infiltrated with distilled water, and the leaves were then centrifuged to remove the fluid. No evidence was presented to show that the fraction obtained by this method contained only intercellular fluid. It seemed possible that it contained material that had been lost from cells damaged during the infiltration and centrifugation processes.

The intercellular fluid isolated by Klement's method supported the growth of phytopathogenic bacteria (3). Its characteristics were therefore of interest as part of a study of the mechanism of induced resistance in tobacco leaves to bacterial diseases (9). The present communication reports the use of enzymic markers to establish the extracellular origin of the fluid. Because plant cell cultures produce large amounts of soluble extracellular peroxidase (8), this enzyme (EC 1.11.1.7) was a possible component of the intercellular fluid. Glucose-6-P dehydrogenase (EC 1.1.1.49), on the other hand, is largely confined to the cytoplasm in several plants (1) and could therefore be used as a marker for contamination of the intercellular fluid by the cytoplasmic contents of leaf cells.

The three largest leaves of a tobacco (*Nicotiana tabacum* L. var. Bottom Special) plant that had been grown in a growth room for about 7 weeks, as previously described (9), were detached, and the laminar, intercostal tissue was excised and cut lengthwise into strips about 1.5 cm wide. An extract was made from half of the strips by cooling them to −79 C with Dry Ice, grinding them in a mortar and pestle and suspending the powder in an ice-cold buffer solution (2 ml for each gram of tissue) of the appropriate type (Table I). The extract was filtered through muslin, centrifuged at 1100*g* for 5 min, and the supernatant fluid was assayed for protein content, peroxidase, and glucose-6-P dehydrogenase activities. The remaining strips were immersed in ice-cold buffer and infiltrated *in vacuo* for three periods of 1 min each. The strips were then removed from the buffer, blotted dry, rolled up, and placed in a 50-ml polypropylene centrifuge tube provided with perforations (2 mm diam) at the bottom. The tube was placed in a 225-ml plastic centrifuge bottle in such a way that the tube was held by its rim and remained suspended in the bottle. The apparatus was centrifuged at 1120*g* (3) for 10 min at 4 C. After centrifugation, the fluid that collected at the bottom of the bottle, and an extract from the leaf tissue that remained in the tube were assayed as above.

Although the intercellular fluid fraction contained less than 1% of the protein present in the original leaf strips, it contained about 45% of the peroxidase (Table I). Thus, the specific activity of peroxidase was 100-fold higher in the intercellular fluid than in the leaf material that remained after centrifugation. The low specific activity of peroxidase in the cell material was not due to the presence of an inhibitor, since the peroxidase activity of a mixture of intercellular fluid and cell extract was equal to the sum of the activities of the two fractions when separate. In the case of glucose-6-P dehydrogenase the specific activity in the residual cell material was higher than in the intercellular fluid. Less than 0.5% of the total activity of the leaf strips was present in the latter.

The higher specific activity of peroxidase in the intercellular fluid fraction suggests that a mechanism exists for the selective export of a soluble form of the enzyme from the leaf cells to the intercellular space. Hence, the appearance of the enzyme in the fluid is not simply the result of diffusion from the cells, whether this be natural or caused by damage to the cells during infiltration and centrifugation. The small amount of glucose-6-P dehydrogenase in the fluid shows that there is very little leakage of cell contents into the intercellular spaces.

The compartmentation of soluble enzymes between the intercellular fluid and the cytoplasm of tobacco leaf cells is reflected in the composition of the intercellular fluid fraction isolated by infiltration and centrifugation. Thus, the method is satisfactory and the fluid may be used for a variety of biochemical and physiological investigations. Preliminary studies

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Table I. *Activity of Enzymes in the Intercellular Fluid Obtained from Tobacco Leaves and in the Residual Cell Material*

For the assay of peroxidase, 0.1 ml of leaf extract or intercellular fluid (in 20 mM sodium phosphate buffer, pH 7.0) was mixed with 2.9 ml of 25 mM sodium acetate buffer, pH 4.5, and 0.05 ml of 20 mM guaiacol. The reaction was started by addition of 0.01 ml of 40 mM H_2O_2, and the initial rate of increase of absorbance at 470 nm was measured. One milliunit of enzyme is the amount causing an absorbance increase of 1.0 per min at 24 C. For the assay of glucose-6-P dehydrogenase, 0.1 M tris-Cl (pH 7.5) was used to prepare intercellular fluid or leaf extract. The assay method was essentially that of Kornberg and Horecker (4), and the results were expressed in international units at 24 C and pH 7.5. Protein was estimated by the method of Lowry *et al.* (5).

	Peroxidase	Glucose-6-P Dehydrogenase	Protein
Leaf strips			
Amount present/g fresh wt	5.3 mU[1]	88.6 mU	15.7 mg
Specific activity (mU/mg protein)	0.3	5.7	
Intercellular fluid			
Amount present/g fresh wt	2.4 mU	0.34 mU	0.12 mg
% of amount in strips	44.9	0.4	0.8
Specific activity (mU/mg protein)	20.0	2.8	
Cell material			
Amount present/g fresh wt	3.1 mU	88.2 mU	15.6 mg
% of amount in strips	57.0	99.5	99.5
Specific activity (mU/mg protein)	0.19	5.7	

[1] mU, milliunits.

have indicated that estimations of the amino acids, phenolic compounds, sugars and electrolytes present in the fluid can readily be made.

Ridge and Osborne (7) reported on insoluble forms of peroxidase, covalently and ionically bound to pea cell walls. In the present work a soluble extracellular peroxidase has been demonstrated in the intercellular fluid of tobacco leaves. Peroxidase catalyzes reactions of lignin (10) and flavonoid (6) synthesis and IAA degradation (2) *in vitro*. As yet, however, there is no firm evidence that it catalyzes any of these reactions *in vivo*. Thus, the importance and function of the high concentrations of extracellular soluble peroxidase in intercellular fluids in leaves cannot be assessed at this time.

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LITERATURE CITED

1. Heber, U., U. W. Hallier, and M. A. Hudson. 1967. Lokalisation von Enzymen des reduktiven und dem oxydativen Pentosephosphat-Zyklus in den Chloroplasten und Permeabilitat der Chloroplasten-Membran gegenüber Metaboliten. Z. Naturforsch. B. 22: 1200–1215.
2. Hinman, R. L. and J. Lang. 1965. Peroxidase-catalyzed oxidation of indole-3-acetic acid. Biochemistry 4: 144–158.
3. Klement, Z. 1965. Method of obtaining fluid from the intercellular spaces of foliage and the fluid's merit as substrate for phytobacterial pathogens. Phytopathology 55: 1033–1034.
4. Kornberg, A. and B. L. Horecker. 1955. Glucose 6-phosphate dehydrogenase. Methods Enzymol. 1: 323–327.
5. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.
6. Rathmell, W. G. and D. S. Bendall. 1972. The peroxidase-catalyzed oxidation of a chalcone and its possible physiological significance. Biochem. J. 127: 125–132.
7. Ridge, I. and D. J. Osborne. 1971. Role of peroxidase when hydroxyproline-rich protein in plant cell walls is increased by ethylene. Nature New Biol. 229: 205–208.
8. Rubery, P. H. 1972. Studies on indoleacetic acid oxidation by liquid medium from crown gall tissue culture cells: the role of malic acid and related compounds. Biochim. Biophys. Acta 261: 21–34.
9. Sequeira, L., S. Aist, and V. Ainslie. 1972. Prevention of the hypersensitive reaction in tobacco by proteinaceous constituents of *Pseudomonas solanacearum*. Phytopathology 62: 536–541.
10. Siegel, S. M. 1970. Biochemistry of the Plant Cell Wall. *In:* M. Florkin and E. M. Stotz, eds., Comprehensive Biochemistry, Vol. 26A. Elsevier, Amsterdam. pp. 1–51.