Specific Localization of β-D-Glucoside Conjugates of 2,4-Dichlorophenoxyacetic Acid in Soybean Vacuoles

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Cell suspension cultures of soybean (*Glycine max* L.) were incubated with [^{14}C]-2,4- dichlorophenoxyacetic acid (62 h, 27 °C). The cells were converted to protoplasts by use of the cell-wall degrading enzymes, Driselase and pectinase. This procedure resulted in the release of the cellular amino acid conjugates as well as part of the free 2,4-dichlorophenoxyacetic acid and of the β-D-glucoside conjugates.

A vacuole fraction was obtained by mild osmotic shock in 10% yield, and was characterized by its polypeptide composition. The purified vacuoles were enriched in β-D-glucoside conjugates, free 2,4-dichlorophenoxyacetic acid being only a minor vacuolar constituent, and amino acid conjugates not being detectable.

Plants are known to metabolize a variety of pesticides and other xenobiotics to polar β-D-glucoside, glutathione and amino acid conjugates [1, 2]. This is superficially similar to the metabolism of xenobiotics in mammalian liver where chemicals are conjugated with D-glucuronic acid, glutathione, or amino acids, to be subsequently excreted via the urinary and biliary excretion pathways. Since plants have no efficient excretion system, it has been proposed that 'local excretion' into the vacuole and into the cell wall may occur [3]. The incorporation of 2,4-dichlorophenoxyacetic acid (2,4-D) into cell wall lignin has recently been demonstrated [4]. However, the proposed deposition of xenobiotic conjugates in the vacuole has apparently never been demonstrated although a great number of endogeneous plant glycosides as well as free amino acids, organic acids and sucrose have been found associated with isolated plant vacuoles [5].

Literature procedures for protoplast and vacuole preparation [6–8] have now been applied to soybean cells which had been incubated with ^{14}C-labeled 2,4-D. The major metabolite fractions formed were amino acid conjugates and β-D-glucoside conjugates [see 9, 10], but only the latter metabolite fraction was associated with the isolated vacuoles.

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Materials and Methods

Materials

The origin and maintainance of the soybean cell suspension culture (*Glycine max* L., cv. Merrill var. Mandarin) has been described previously [10]. The enzyme preparation Driselase was used as supplied by Fluka (Neu-Ulm, FRG). The pectinase preparation (Sigma, St. Louis, No. P-5146) was dialyzed against excess water using cellulose-free dialysis bags (Naturin Co., Weinheim, FRG). The enzyme was employed after lyophilization. Nylon cloth with 70 µ or 50 µ mesh size was obtained from Eckert Co., Waldkirch, FRG. Other materials were as previously described [4, 10, 11].

General methods

The following methods have previously been described, determination of protein and radioactivity, sodium dodecylsulphate (SDS) gel electrophoresis [4, 10, 11], incubation of soybean cells with [2-^{14}C]-2,4-D (4 µCi, 62 h) [10], digestions with β-glucosidase and proteinase K [10]. Staining with neutral red was by the procedure of Sitte [12].

A simplified procedure of metabolite extraction from protoplast or vacuole suspensions consisted in adding 3.75 parts chloroform/methanol, 1:2 v/v, to 1 part aqueous suspension. After thorough mixing, insoluble material was isolated by centrifugation and extracted twice with 1–2 parts chloroform/methanol,

1:2 v/v. The combined extracts were directly employed for chromatographic analysis.

Protoplast preparation

The procedure of Galbraith and Northcote [7] was modified as follows. The soybean cells were harvested by filtration 7 days after inoculation. [2-^{14}C]-2,4-D had been added to the growing culture 62 h prior to harvest [see 10]. The yield of cells was 10–12 g wet weight from 40 ml medium. A portion of the cells (5–6 g) was suspended in 6 ml buffer I (0.35 M mannitol, 0.35 M sorbitol, 6 mM $CaCl_2$, 1 mM $CaHPO_4$, pH 6.5). After 5 min at 25 °C, 180 mg Driselase and 60 mg pectinase was added. These enzymes had been dissolved in 1.3 ml, buffer I, and insoluble material had been removed by centrifugation. The cell suspension was incubated with constant shaking (120 rpm, 28 °C), and microscopic control. After complete conversion to protoplasts (usually after about 2 h) the suspension was filtered into a centrifuge tube through 70 μ nylon cloth. Subsequently, 6 ml 22% (w/v) Ficoll (Pharmacia, Uppsala, Sweden) in buffer I was layered underneath the suspension, followed by centrifugation (5000 rpm 10 °C, 30 min) in the HB-4 Sorvall rotor. The protoplasts formed a thick white layer on top of the Ficoll layer whereas cellular debris sedimented to the bottom of the centrifuge tube. The supernatant was carefully removed. The protoplast layer was suspended in 5 ml buffer I and transferred to another centrifuge tube. The protoplasts were sedimented in a low-speed centrifuge (Labofuge Christ, position 3, appr. $100-200 \times g$, 5 min, 25 °C). The protoplasts were washed 4 more times by the same procedure, to be finally suspended in 2 ml buffer I.

Vacuole preparation

The methods of Wagner and Siegelman [8] and Boller and Kende [6] were modified as follows. Protoplast suspension (1 part) was added to 10 parts buffer II (0.1 M K_2HPO_4, 1 mM dithiothreitol, pH 8.0), followed by incubation with constant shaking (120 rpm, 20 min, 28 °C). The spontaneous release of vacuoles was followed by phase-contrast microscopy. The suspension was filtered into a centrifuge tube through 50 μ nylon cloth, and underlayered with 2 ml of 10% (w/v) Ficoll in a mixture of 1 part buffer I and 10 parts buffer II. The vacuoles were pelleted onto the Ficoll layer by centrifugation (Sorvall HB-4 rotor, 5500 rpm, 30 min, 10 °C). The supernatant was carefully removed and the vacuole layer was immediately used for further characterization.

Results

Vacuole preparation

The protoplast preparation procedure used led to $20-25 \times 10^6$ protoplasts from 10 g wet cells, as determined in a counting chamber. The washed protoplasts had diameters of 20–40 μ, and were well dispersed (see Fig. 1 A). The protoplasts were intensely stained with neutral red in a yield of close to 100%. Several methods for the release of vacuoles were tested. The best yield of about 10% was obtained with the procedure of Wagner and Siegelman [8], as modified by Boller and Kende [6]. This procedure led to the virtual disappearance of protoplasts, but particulate cellular material adhered to the liberated vacuoles (Fig. 1 B). Filtration through 50 μ nylon cloth and centrifugation onto a Ficoll layer resulted in some purification of the vacuoles (Fig. 1 C). The vacuoles were about 30–40 μ in diameter, and could not be stained with neutral red by the procedure of Sitte [12]. The vacuoles were extremely sensitive to osmotic stress but they remained intact when stored for several hours at 8 °C.

The protoplast and the vacuole preparations were characterized by SDS-gel electrophoresis (Fig. 2). The protoplasts contained some polypeptides which possibly originated from the Driselase and pectinase preparations used. Such polypeptide bands are marked in Fig. 2 A. Most of the polypeptide bands of the vacuole preparation differed from those of the protoplasts. A crude tonoplast fraction was prepared from the isolated vacuoles, and a distinctly different polypeptide composition was noted (Fig. 2 B). A strong polypeptide band with an apparent molecular weight of about 17 000 was present in the protoplast and vacuole preparations. This polypeptide was virtually absent from the tonoplast fraction. Polypeptides of the following approximate molecular weight values were enriched in the tonoplast fraction relative to the soluble vacuolar protein fraction, 70 000, 62 000, 60 000, 35 000 and 32 000 (see Fig. 2 B). The molecular weight values of the most intense polypeptide bands of *Hippeastrum*

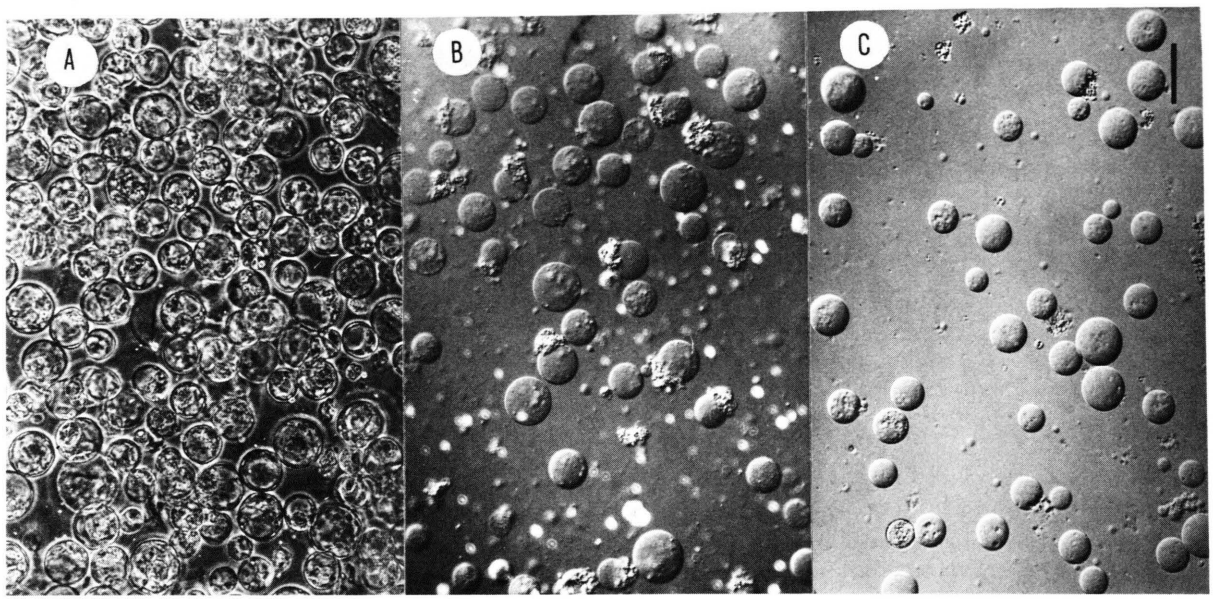

Fig. 1. Microscopic appearance of A) the washed soybean protoplasts, B) the vacuolar fraction after release from the protoplasts and C) the vacuolar fraction after filtration and centrifugation. A Zeiss Photomikroskop II was used with Nomarski optics. The length of the bar in Fig. 1 C corresponds to 50 μ. Panels A, B and C are at the same magnification.

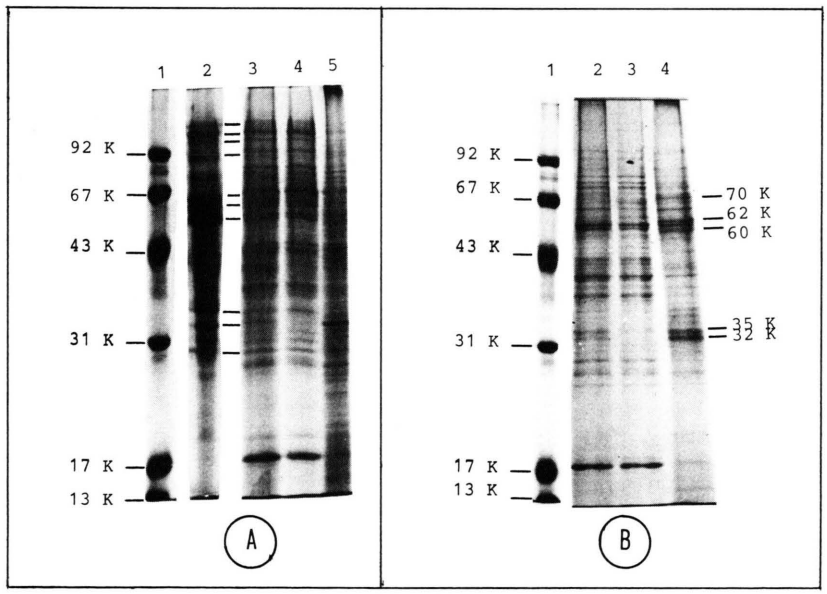

Fig. 2. A. SDS gel electrophoretic patterns obtained with the following samples. 1. Reference proteins, phosphorylase b (92 K), bovine serum albumin (67 K), ovalbumin (43 K), carboanhydrase (31 K), myoglobin (17 K) and cytochrome c (13 K). 2. Driselase and pectinase enzyme mixture. 3. Washed protoplast preparation of Fig. 1 A. Polypeptides which possibly originated from the Driselase and pectinase mixture are marked. 4. Soluble protein fraction of the protoplast preparation. 5. Particulate protein fraction of the protoplast preparation. B. SDS gel electrophoretic patterns obtained with the following samples. 1. Reference proteins as in Fig. 2 A. 2. Vacuole preparation of Fig. 1 C. 3. Soluble protein fraction of the vacuole preparation. 4. Particulate protein fraction of the vacuole preparation. The soluble and particulate protein samples were prepared as follows. The protoplast or vacuole suspension was sonicated (Branson sonifier B-12, microtip, 5 sec, 25 °C), followed by ultracentrifugation (100 000 g, 2 h, 4 °C). The supernatant was employed as soluble protein fraction, whereas the pellet material was used as particulate protein fraction.

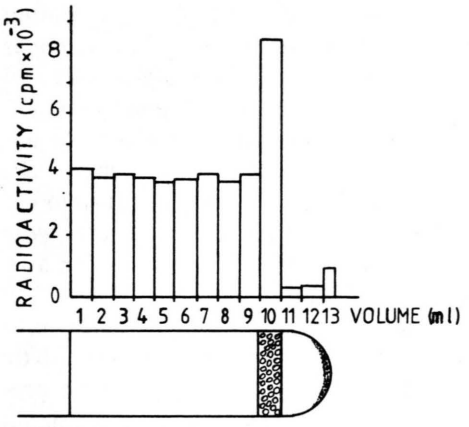

Fig. 3. Distribution of radioactivity upon centrifugation of the vacuole fraction released from soybean protoplasts by mild osmotic shock. Individual fractions were collected successively from the centrifuge tube by means of a peristaltic pump, and aliquots were used to determine radioactivity.

tonoplast have been reported as 69 000, 59 000 and 53 000, respectively [13].

Distribution of 2,4-D metabolites

After incubation with [2-^{14}C]-2,4-D for 62 h, about one third of the radioactivity was present in the culture medium. This material consisted to about 97% of unchanged 2,4-D [10]. Two thirds of the initial radioactivity was associated with the soybean cells, but about 90% of this amount was released from the cells during the preparation and washing of the protoplasts. Nevertheless, an enrichment of radioactive material in the vacuole band was found (Fig. 3).

The chemical nature of the various radioactive fractions was studies by the previous methods [10], with the exception of the cellular material removed in the two filtration steps. The radioactive material

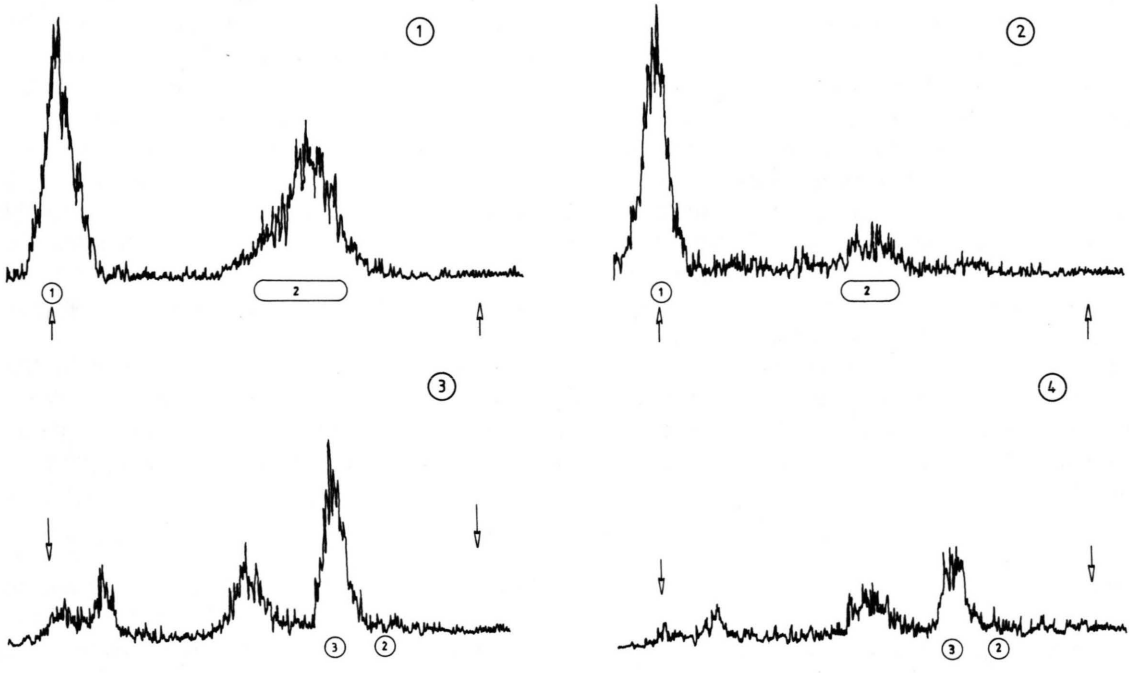

Fig. 4. Distributions of radioactivity on thinlayer chromatograms of the following samples. 1. Total protoplast extract. 2. Total vacuole extract. 3. Chromatographically immobile material of sample 1 after methanol elution and β-glucosidase treatment. 4. Chromatographically immobile material of sample 2 after methanol elution and β-glucosidase treatment. The extracts of samples 1 and 2 were prepared by the simplified extraction procedure of Materials and Methods. Thinlayer chromatography was performed on pre-coated silica gel G plates (Merck No. 5554) using the following solvent system, diethyl ether/petrol ether (40–60 °C)/formic acid, 50:50:2, v/v/v. The positions of the following reference compounds are shown, 1. β-D-glucoside of 4-hydroxy-2,5-dichlorophenoxyacetic acid, 2. 2,4-D, 3. 4-hydroxy-2,5-dichlorophenoxyacetic acid. The radioactivity profiles were obtained using a Berthold model LB 2821 automatic scanner with integrator unit. The arrows refer to the origin (left) and the front of the chromatograms.

released during preparation and washing of the protoplasts contained free 2,4-D (85–90%), its 4-hydroxy derivatives (about 5%) and polar conjugate material (6–10%). The high amounts of free 2,4-D and its 4-hydroxy derivatives were probably due to the cell-wall degrading enzymes used. The Driselase and pectinase enzyme preparations were highly active in the hydrolysis of the purified β-D-glucoside and amino acid conjugate fractions formed from 2,4-D by the soybean cells [results not shown in detail].

The conjugate material released from the cells during preparation and washing of the protoplasts was further studied after ether extraction [10] and chromatographic purification in the solvent system of Fig. 4. The conjugate fraction released unchanged 2,4-D as the only product of acid hydrolysis (2 N HCl, 80 °C, 20 h) as well as alkaline hydrolysis (0.1 N NaOH, 80 °C, 20 h). The conjugate fraction was resistant against purified β-glucosidase as well as proteinase K. On the basis of previous results [10], the conjugate fraction therefore appeared to consist exclusively of amino acid conjugates.

The isolated protoplast and vacuole preparations contained unchanged 2,4-D as well as a polar metabolite fraction (Fig. 4). Free 2,4 D was a minor constituent of the vacuoles (13%), but it accounted for 51% of the radioactivity of the protoplasts. The polar metabolite fractions were eluted from the thinlayer chromatograms. These metabolites were cleaved by purified β-glucosidase (Fig. 4). The major aglycone liberated co-chromatographed with authentic 4-hydroxy-2,5-dichlorophenoxyacetic acid. The minor aglycone liberated was probably identical to 4-hydroxy-2,3-dichlorphenoxyacetic acid [9], but no authentic standard was available. No unchanged 2,4-D was liberated from the β-D-glucoside fraction (see Fig. 4).

Discussion

The above results demonstrate the specific localization of β-D-glucoside conjugates derived from 2,4-D in soybean vacuoles. This result could be obtained even though a considerable efflux of 2,4-D conjugates occurred during protoplast preparation and the external Driselase and pectinase enzymes were highly active in cleaving the β-D-glucoside and amino acid conjugates of 2,4-D. It should be noted that free 2,4-D has previously been shown to undergo faster efflux from intact cultured soybean cells than the amino acid conjugates of 2,4-D [14]. In the present case, however, amino acid conjugates were virtually absent from the washed protoplasts, whereas some free 2,4-D was retained. The efflux occurring during protoplast formation may have been due to osmotic stress or to the presence of lytic impurities in the cell-wall degrading enzyme preparations. The cellular vacuoles appeared to remain intact during protoplast formation, as judged from the positive neutral red reaction of the protoplasts.

The radioactivity of the washed protoplasts (about 44 000 cpm in the experiment of Fig. 3) could be entirely attributed to the vacuolar compartment when the yield of vacuole formation of 10% was taken into account. The excess radioactivity of the vacuole band of Fig. 3 amounted to 4200 cpm above background so that the remaining radioactivity could be attributed to broken and leaky vacuoles.

In conclusion, the present results appear to be best interpreted in terms of efflux and vacuolar retention, respectively. The amino acid conjugates showed complete efflux from the protoplasts, whereas free 2,4-D and its β-D-glucoside conjugates were partially retained in the vacuole. It remains possible that the amino acid conjugates were originally also present in the cellular vacuole but had a considerably higher rate of efflux.

In any case, the present study appears to be the first report of the vacuolar localization of a xenobiotic conjugate so that the previous metabolic scheme [4] receives further experimental support.

Acknowledgements

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[1] G. L. Lamoureux and D. S. Frear, in: Xenobiotic metabolism. *In vitro* methods (G. D. Paulson, D. S. Frear and E. P. Marks, eds.), ACS Symposium Series No. 97, pp. 77–128, American Chemical Society, Washington, D.C. (1979)
[2] H. Sandermann, in: Environmental mutagenesis, carcinogenesis and plant biology (E. J. Klekowski, ed.), Praeger Scientific, New York, in press.
[3] H. Reznik, Erg. Biol. **23,** 14–46 (1960).
[4] D. Scheel and H. Sandermann, Planta **152,** 253–258 (1981)
[5] F. Marty, D. Branton, and R. A. Leigh, in: The biochemistry of plants, Vol. 1 (N. E. Tolbert, ed.), pp. 625–658, Academic Press, New York 1980
[6] T. Boller and H. Kende, Plant Physiol. **63,** 1123–1132 (1979)
[7] D. W. Galbraith and D. H. Northcote. J. Cell Sci. **24,** 295–310, (1977).
[8] G. J. Wagner and H. W. Siegelman, Science **190,** 1298–1299 (1975)
[9] R. O. Mumma and R. H. Hamilton, in: Xenobiotic metabolism. *In vitro* methods (G. D. Paulson, D. S. Frear, and E. P. Marks, eds.), ACS Symposium No. 97, pp. 35–76, American Chemical Society, Washington, D.C., 1979
[10] D. Scheel and H. Sandermann, Planta **152,** 248–252 (1981)
[11] H. Diesperger and H. Sandermann, Planta **146,** 643–648 (1979)
[12] P. Sitte, Biologie in unserer Zeit **2,** 192–194 (1972)
[13] G. J. Wagner, Plant Physiol. **68,** 499–503 (1981)
[14] M. J. Montague, R. K. Enns, N. R. Siegel, and E. G. Jaworski, Plant Physiol. **67,** 603–607 (1981)