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Short Communication

Isolation of Mitochondria from Soybean Leaves on Discontinuous Percoll Gradients[1]

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ABSTRACT

A technique to isolate mitochondria from chamber-grown soybeans (*Glycine max* cv Williams) was developed. The mitochondria were isolated by centrifugation on discontinuous Percoll gradients which yielded a sharp band of mitochondria contaminated by only 4% of the total chlorophyll in the gradient. Contamination by peroxisomes was also slight. The isolated mitochondria oxidized malate plus glutamate, NADH, and malate with respiratory control. They also showed cyanide-insensitive, alternative pathway activity which was inhibited by salicylhydroxamic acid.

Isolation and purification of mitochondria from green leaf tissue is always a challenge due to the chloroplasts cosedimenting with the mitochondria. For example, in sucrose gradients the density of whole plastids is 1.21 to 1.24 g/cm^3, the density of intact mitochondria is 1.18 to 1.20 g/cm^3, and the density for thylakoid fragments from broken plastids is 1.16 to 1.18 g/cm^3 (16). This distribution of plastid densities overlaps the mitochondria and, therefore, the best results are obtained from an isolation technique which does not disrupt chloroplast integrity.

Leaf mitochondria which are free from chloroplasts have been isolated from a variety of species using sucrose density gradients (14), Percoll density gradients (10, 11), two polymer aqueous phase partition (9), or a combination of these techniques (3). Mitochondria have also been isolated from leaf protoplasts (15).

Sucrose gradients, however, may not provide adequate separation of organelles in plants which have a high starch content as the plastids increase in density. Aqueous phase partition and the use of protoplasts, although perhaps the most gentle methods of organelle isolation, may have long separation times (*e.g.*, 3 h), and in the case of phase partition the isolated mitochondria tend to be contaminated by peroxisomes (1).

In this paper, we describe a method developed to isolate mitochondria from the leaves of chamber-grown soybeans. The procedure is successful for all ages of leaf tissue, and for tissue of varying starch content. The mitochondrial band was almost pure with only slight contamination of chloroplasts and peroxisomes as indicated by marker enzymes. As far as we can determine, this is the first report describing the isolation and purification of metabolically functional mitochondria from soybean leaves.

MATERIALS AND METHODS

Plant Material. Soybeans (*Glycine max* cv Williams) were planted in 15-cm pots containing vermiculite and were grown in Environmental Growth Chambers at 25°C, with a 12-h photoperiod. The plants were illuminated with a mixture of fluorescent and incandescent bulbs at 600 μE/m^2·s at pot height. They were flushed daily with a nutrient solution developed by Dr. F. W. Snyder (of the Plant Photobiology Lab) containing (mM): $Ca(NO_3)_2 \cdot 4H_2O$, 4.0; KNO_3, 4.0; $NH_4H_2PO_4$, 1.0; NH_4Cl, 1.5; KCl, 2.5; K_2SO_4, 1.0; and $MgSO_4 \cdot 7H_2O$, 3.5. Micronutrient concentrations were (μM): H_3BO_3 20.6; $CuSO_4 \cdot 5H_2O$, 0.16; $MnSO_4 \cdot 2H_2O$, 4.5; $(NH_4)_6Mo_7O_{24}$, 0.07; $ZnSO_4 \cdot 7H_2O$, 0.34; and Fe-chelate, 107.4 as Sequestrene 330 (Ciba-Geigy Corp).[2]

Mitochondrial Isolation. Mitochondria were isolated from trifoliolate 4 at 18 d postemergence and trifoliolate 5 (numbered from the ground up) at 20 d postemergence. At this time, the leaves were still enlarging and were about two-thirds of the fully expanded size. Approximately 10 to 15 g of leaf tissue were rinsed in distilled H_2O, dried, and the midveins removed. The leaves were cut with scissors into 50-ml cold grinding medium consisting of the following: 0.3 M sorbitol, 50 mM Hepes (pH 7.5 w/KOH), 1 mM EDTA, 1 mM $MgCl_2$, 10 mM DTT, 1% PVP-40 (w/v), and 0.4% BSA (w/v). The tissue was then chopped with sharp razor blades on ice for 5 min and homogenized with a Polytron (setting 4) for 4 s. The Polytron blade was rinsed in 20 ml cold grinding medium, and the brei was filtered through two layers of Miracloth and two layers of 20-μm mesh nylon netting wetted with 30 ml cold grinding medium.

The filtrate was centrifuged for 10 min at 1500*g* in a Dupont Sorval RC-5 centrifuge to remove whole cells and other heavy debris. Depending on the amount of original tissue, 10 to 15 ml of the resulting supernatant was layered on top of a discontinuous Percoll gradient consisting of the following steps: 5 ml each 15%, 22%, and 27%; and 7 ml 60% (v/v). Each step also contained 0.25 M sucrose, 1 mM KH_2PO_4, 10 mM Hepes (pH 7.5 w/KOH), and 0.1% BSA (w/v). The gradients were then spun in a Beckman L5-65 ultracentrifuge with a SW-27 swinging-bucket rotor at

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21,000 rpm for 45 min. After centrifugation, the gradient was fractioned from the top by an ISCO Density Gradient Fraction Collector model 640 using a 56% (w/w) sucrose solution.

Oxygen Electrode Measurements. Oxygen uptake was determined polarographically with a Clark-type O_2 electrode at 25°C. A sample of 800 μl mitochondrial fraction was added to 200 μl reaction medium consisting of 0.3 M sucrose, 10 mM Hepes (pH 7.2 w/KOH), 10 mM KCl, 5 mM $MgCl_2$, 10 mM KH_2PO_4, and 0.1% BSA (w/v). Additions were made yielding final concentrations of 10 mM DL-malate in 10 mM Hepes-KOH (pH 7.2), 10 mM L-glutamate in 10 mM Hepes-KOH (pH 7.2), 2.5 mM NADH, 0.05 mM ADP, 1.8 μg/ml Antimycin A, 1 mM KCN, 1 mM SHAM,[3] and 0.04 mM rotenone. The final volume after additions was 1.15 ml. ADP:O ratios were calculated by the method described in Estabrook (8). This ratio is defined as μmol of ADP added, divided by the μatoms oxygen utilized during state 3 respiration. Addition of ADP causes an increase in the oxygen utilization and transition to state 3 respiration—not limited by ADP or substrate.

Organelle Marker Enzyme Assays. NAD^+-dependent isocitrate dehydrogenase was assayed by the method of Cox (6) modified by the addition of 0.5 mM KCN and 0.01% Triton X-100. Cyt *c* oxidase was measured by the method of Simon (17). Catalase was measured by the method of Lück (13) and hydroxypyruvate reductase by the method of Liang *et al.* (12) modified by the addition of 0.5 mM KCN. Chl was extracted in 100% methanol and determined by the method of Cosio *et al.* (5). Protein was determined by the following procedure. Mitochondria were diluted in an isotonic solution and pelleted using a Beckman Microfuge-12 to remove the BSA. The supernatant was discarded and the pellet rinsed, resuspended, and repelleted. This final pellet was resuspended in a known volume and the protein measured using Coomassie blue (4) with BSA as a standard.

RESULTS AND DISCUSSION

The isolation procedure resulted in the gradient profile seen in Figure 1. The mitochondria were well separated from the chloroplasts as well as broken thylakoid fragments. The mitochondria were discernable in the centrifuge tube as a white band at the interface of the 27% and the 60% Percoll layers with no visible chloroplast contamination. The slight chloroplast contamination seen in the mitochondrial fraction occurred during fractionation when some clumped thylakoids clinging to the side of the polyallomer tube became mixed with the mitochondrial band as it moved up the tube. Others have experienced the same problem when fractioning from the top (14). This technique was also successful for isolating mitochondria from CO_2-enriched soybeans which have a greater plastid density due to a higher starch content. No difference was seen in the activities of CO_2-enriched mitochondria and controls (unpublished).

The mitochondria were concentrated in one fraction, as detected by the two mitochondrial marker enzymes NAD^+ isocitrate dehydrogenase and Cyt *c* oxidase. The dehydrogenase activity varied from 24.1 to 40.2 nmol/min·ml depending on the amount of tissue used for each preparation. The activity of Cyt *c* oxidase varied from 129.3 to 238.8 nmol/min·ml. The specific activities were 0.8 μmol/min·mg protein and 2.7 μmol/min·mg protein for isocitrate dehydrogenase and Cyt *c* oxidase, respectively. Isocitrate dehydrogenase exhibited increased activity with the addition of Triton X-100, indicating that the mitochondria were intact.

Catalase activity was found mainly in the supernatant fractions. To determine if the activity was due to cytoplasmic or

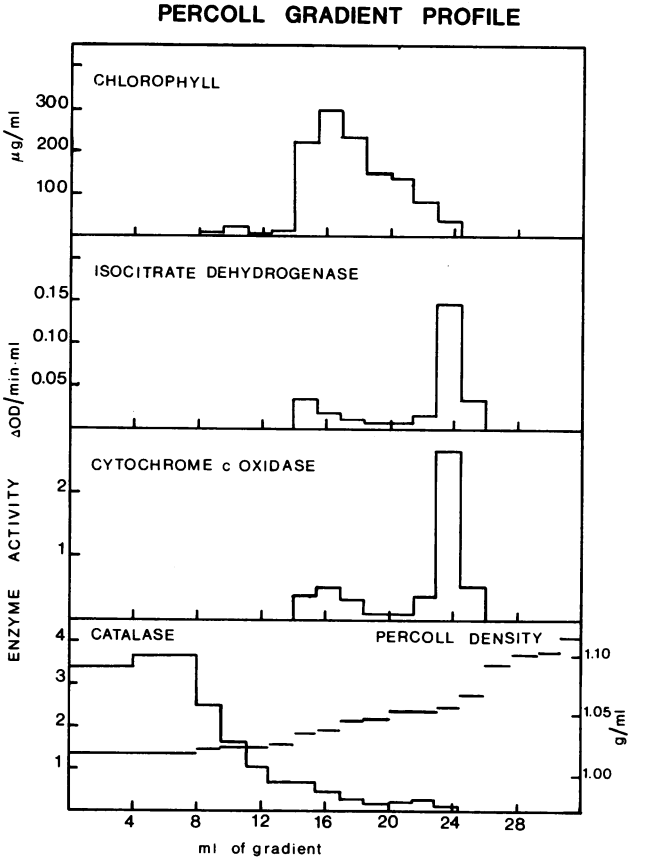

FIG. 1. Discontinuous Percoll gradient profile showing the distribution of organelles as determined by marker enzymes. The gradient consisted of 10 ml of soybean leaf homogenate (from leaf 4) layered on to the following steps: 5 ml each 15%, 22%, and 27%; and 7 ml 60% (v/v). The gradient was centrifuged at 21,000 rpm for 45 min. The refractive index of Percoll was determined by a refractometer and converted to Percoll density as described in the Percoll Handbook, published by the manufacturers, Pharmacia Fine Chemicals.

Table I. *State 3, State 4, Respiratory Control, and ADP:O Ratios for Malate and Glutamate, NADH, and Malate-Dependent Oxygen Uptake*

The following concentrations were used: 10 mM DL-malate, 10 mM L-glutamate (as described in "Materials and Methods"), and 2.5 mM NADH. Additions of ADP were 0.03 to 0.05 mM.

Substrate	State 3	State 4	RC	ADP:O
	nmol/min·mg protein		*ratio*	
Malate, glutamate	169.88	147.75	1.15	3.56
NADH	158.62	84.39	1.88	4.31
Malate	27.93	18.07	1.55	8.88

peroxisomal catalase, hydroxypyruvate reductase was assayed. The distribution of hydroxypyruvate reductase followed the catalase distribution closely (data not shown), indicating that most of the peroxisomes were broken during the isolation procedure (Fig. 1).

The mitochondria exhibited malate plus glutamate-dependent and NADH-dependent O_2 uptake at a much greater rate than malate-dependent O_2 uptake (Table I). These values are similar to those reported for spinach (3, 9) and pea (14). The respiratory controls were slightly lower than those found for other species; however, this may be due to the age of the leaf tissue. The tissue used for the polarographic studies was from trifoliolate 5 which was still expanding and about two-thirds full size. It has been reported that young plants do not have tightly coupled mito-

[3] Abbreviation: SHAM, salicylhydroxamic acid.

Table II. *Per Cent Inhibition of Malate and Glutamate, NADH, and Malate-Dependent Oxygen Uptake by Mitochondrial Inhibitors*

Rates are expressed as per cent inhibition of state 3 rates (see Table I) following addition of the inhibitor, or sequential addition of inhibitors. The following concentrations were used: 1 mM KCN, 1 mM SHAM, 0.04 mM rotenone, and 1.8 μg/ml antimycin A.

Additions	Malate Glutamate	NADH	Malate
		%	
KCN	70.0	100	
followed by SHAM	96.5		
Rotenone		10.3	11.8
followed by antimycin A		98.3	25.0
followed by SHAM			100

chondria (18). The apparent ADP:O ratio for malate is unusually high. This is perhaps due to the slow oxidation of malate in the absence of glutamate. The presence of glutamate allows the transamination of the oxalacetic acid formed, and the malate dehydrogenase reaction proceeds in the forward direction.

Malate and glutamate oxidation was inhibited 70% (Table II) by the addition of KCN. The addition of SHAM at this point stopped the reaction completely. Therefore, the alternative pathway activity in these young leaves comprises 30% of the total oxidation activity. Azcon-Bieto *et al.* (2) reported that the percentage of cyanide-resistance is less in younger tissues. Exogenous NADH oxidation was inhibited by rotenone only 10.3%, but totally inhibited by subsequent addition of Antimycin A. This is consistent with the fact that external NADH is oxidized by an externally facing, inner membrane NADH dehydrogenase which bypasses the rotenone-sensitive site and the first phosphorylation site (7).

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