An abbreviated method for the isolation of mitochondria from storage tissues of oil seedlings

Douglas G. Luster and Roger C. Fites


High yields of metabolically active mitochondria can be rapidly isolated from reserve storage tissues of castor bean (Ricinus communis L.), cucumber (Cucumis sativus L., cv. Early Green Cluster), cotton (Gossypium hirsutum L., cv. Coker 711), and soybean (Glycine max L., cvs Forrest and Centennial) seedlings by application of crude homogenates to discontinuous sucrose gradients, allowing for a rapid separation of organelles from detrimental factors present in cell homogenates.

Additional key words – Fatty acids, mitochondrial integrity, phenolics, respiratory control ratios.

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Introduction

The storage tissues of germinating oil seedlings contain active lipases (Liu et al. 1982, Nishimura and Beever 1979) and proteases (Alpi and Beever 1981) which can alter the integrity and activity of membrane preparations isolated from these tissues. Polyphenolic compounds, which are known to alter mitochondrial activities (Loomis 1974), may also be sequestered in reserve tissues, most notably in the resin glands of cotton cotyledons. In addition, lipidic materials, primarily fatty acids, which are detrimental to electron transport and oxidative phosphorylation in isolated mitochondria (Delgarno and Birt 1963), are released upon homogenization of these tissues.

We report here an isolation scheme which relies upon the direct application of crude homogenates to discontinuous sucrose gradients, excluding differential centrifugation steps traditionally performed prior to gradient loading in order to achieve a more rapid separation of mitochondria from degradative factors in cell homogenates.

Abbreviations – ADP:O ratio, ADP:O oxygen consumption ratio; BSA, bovine serum albumin; Cyt. oxidase, cytochrome c oxidase; DTE, dithioerythritol; EGTA, ethyleneglycol bis(β-aminocetyl ether)-N,N,N',N'-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; NADP⁺, 6-P-G DH, 6-P-glucanate:NADP⁺ dehydrogenase; NADPH:CCR, NADPH:cytochrome c reductase; RC, respiratory control; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

Materials and methods

Mitochondrial isolation

The isolation procedure used is a modification of the method developed by Beever and his colleagues for isolation of glyoxysomes (Cooper and Beever 1969) and mitochondria (Chappell and Beever 1983) from castor bean endosperm. Castor bean (Ricinus communis L.) and cotton (Gossypium hirsutum L., cv. Coker 711) seedlings were grown in moist paper towel scrolls, while cucumber (Cucumis sativus L., cv. Early Green Cluster) and soybean (Glycine max L., cv. Forrest and Centennial) seedlings were grown in moist vermiculite. Seedlings were grown in a dark incubator at 23 to 30°C for 3 to 6 days. Endosperm obtained from seedlings of castor bean, or cotyledons from cucumber, cotton and soybean were used as the tissue sources. In order to pro-

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duce high yields of mitochondria, the tissue fresh weight to homogenization medium volume ratio during homoge-

nization was ca 1:1 (w/v); consequently the concen-

tration of osmoticum (sucrose) was kept at 0.6 M to off-

set the dilution caused by such a high tissue to volume ratio. This was particularly important in tissues with a high water content (e.g. castor bean endosperm). Ten to forty g of seedling reserve tissue were hand-chopped into 5 mm pieces using multiple razor blades and subse-

quently ground to a thick paste for 10 min in a mortar and pestle in one volume of homogenization medium composed of: 0.6 M sucrose, 150 mM TES-KOH, pH 8.0, 1.0 mM EGTA, 10 mM KCl, 0.1 mM MgCl₂, 0.25% (w/v) defatted BSA, 2.0 mM Na₃S₂O₃, and 10 mM DTE. The brei was filtered through two layers of 40-80 µm,

mesh nylon parachute cloth and 10 to 13 ml (150-250 mg protein) of the filtrate quickly layered onto a 19 ml discon-tinuous sucrose gradient consisting of: 4.0 ml 50%, 5.0 ml 45%, 3.0 ml 35% and 2.0 ml 32% (w/v) sucrose in 10 mM HEPES-KOH, pH 7.2, containing 0.1% (w/v) defatted BSA. Thus, regions sur-

rounding the interface at which mitochondria were ex-

pected to band (40-50%) were expanded in volume to allow maximal homogenate loading while avoiding o-

ganellar band overlap. The loaded gradients were then cen-trifuged for 2 h at 22,000 x g (R₂₀ = 73,000 g) in a Beckman Model L ultracentrifuge using an SW 25.1

swinging bucket rotor. Following centrifugation, gra-

dients were either fractionated into 1.0 ml fractions and as-

sayed for marker enzymes or previously identified mi-

tochondrial bands were aspirated for further character-

ization. After aspiration with a chilled syringe, mito-

chondrial fractions were very slowly diluted (1.0 ml min⁻¹) with 2 volumes of 0.25 M sucrose in 10 mM TES-

KOH, pH 7.2, containing 0.1% (w/v) defatted BSA, and pelleted at 10,000 x g for 20 min. Mitochondrial pel-

lets were then typically suspended in the same medium to produce a final concentration of 5 to 10 mg mi-

tochondrial protein ml⁻¹ for use in enzyme assays or measurement of oxygen consumption. All operations were performed at 0-4° C.

Oxygen consumption

Mitochondrial oxygen uptake was measured polaro-

graphically in a 2 ml stirred vessel with an oxygen elec-

trode (Yellow Springs Instruments) at 25°C. The mi-
dium contained 0.25 M sucrose, 20 mM KH₂PO₄-KOH, pH 7.1, 10 mM KCl, 0.5 mM MgCl₂, and 0.1% (w/v) de-
fatted BSA. Mitochondria (ca 0.10 to 0.25 mg protein

ml⁻¹) were preincubated for 10 min with 0.5 mM ATP prior to addition of substrate (10 mM). Estimates of oxygen uptake and respiratory control and ADP/O ra-

tios were made according to Estabrook (1967), using only those rates obtained following the completion of three successive cycles of depletion of added ADP (30 µM), to allow for completion of the “conditioning” phe-

nomenon (Raison et al. 1973).

Enzyme assays

Catalase (EC 1.11.1.6; Luck 1963), sumarase (EC

4.2.1.2; Cooper and Bevers 1969), NADPH dehydrogenase (EC 1.1.1.44; Simcox et al. 1977) and ribulose bisphosphate carboxylase-oxygenase (EC 4.1.1.39; Nishimura et al. 1976) were assayed as described. NADPH-cytochrome c reductase (Strobel and Dignam 1978) assays included KCN (1 mM) and antimycin A (10 µM). Isocitrate lyase (EC 4.1.3.1) was assayed following the procedure of Dixon and Korning (1959), substituting glyoxylic acid for phosphate and DTE for cysteine. Measurements of outer mitochondrial membrane intactness using an atomic absorption c reductasc were conducted according to the procedure of

Douce et al. (1972). Enzyme assays were conducted at room temperature (23-25°C).

Protein content in gradient fractions and isolated o-

ganelles was assayed by the methods of Bradford (1976)

and Markwell et al. (1981), respectively.

Results and discussion

Mitochondria isolated from oil seedling reserve tissues in our laboratory using protocols incorporating lengthy differential centrifugations such as that of Bonner

(1967) or Douce et al. (1972), or the shortened method of Chappell and Bevers (1983) exhibited weak coupling of substrate oxidation to phosphorylation and less than 50% outer membrane intactness (data not shown). Abbreviated methods incorporating rapid differential centrifugation of mitochondria to preserve intactness (e.g. Palmer 1967) resulted in considerable contami-

nation of mitochondrial fractions with glyoxysomes (data not shown). Figure 1 represents a typical marker enzyme profile obtained upon fractionation of a discontinuous sucrose gradient loaded with castor bean endosperm homoge-

nate. Mitochondria, located by cyt. c oxidase, typically banded just above the 40/45% sucrose gradient inter-

face regardless of species or cultivar, while glyoxy-

somes, located by catalase, were invariably loosely pel-

leted at the bottom of the gradient (Fig. 1). These marker enzymes were well separated, with minimal overlap of either enzymatic activity in fractions located between the peaks of activity. Catalase activity was present throughout the gradients at low levels, with the highest concentrations located in the homogenate re-

gion (Fig. 1), probably due to breakage of the fragile glyoxysomes during homogenization, and in the glyoxy-

somal fractions (Fig. 1). The contamination by glyoxy-

somes was estimated from catalase specific activity to be

2% in the peak mitochondrial fraction.

The endoplasmic reticulum was located by assaying for NADPH-cyt. P-450 reductase using cyt. c as electron acceptor (= NADPH CCR) in the presence of antii-

mycin A and cyanide to exclude contribution by the mi-

tochondrial inner membrane. NADPH CCR was pres-
ent near the interface between the cytosolic fraction and the sucrose gradient, and in the glyoxysomal fraction at the bottom of the gradient (Fig. 1). A small peak of activity was located in the mitochondrial fraction as well. DTE included in the homogenization buffer interfered with the measurement of cyt. c reduction in fractions located near the gradient interface, making calculations of specific activity unreliable in such fractions. However, the maximum specific activity of this enzyme measured in mitochondrial fractions aspirated and pellet from gradients [ca 10 nmol (mg protein)⁻¹ min⁻¹; data not shown], was 10% of the typical, found in similarly purified endoplasmic reticulum fractions from castor bean endosperm of this age [ca 100 nmol (mg protein)⁻¹ min⁻¹; D. G. Luster and R. P. Donaldson, unpublished data].

Protoplasts were located on the gradients using 6-P-gluconate:NADP⁺ dehydrogenase, which is also present in the cytosol of castor bean endosperm cells (Nishimura and Bevers 1979). The enzyme sedimented in a broad peak with the glyoxysomes, and was also present in the cytosolic fraction above the sucrose gradient (Fig. 1). Ribulose bisphosphate carboxylase-oxygenase, used by Nishimura and Bevers (1979) to locate proplastids in gradient-fractionated castor bean endosperm protoplasts, was undetectable in aspirated mitochondrial fractions. Carotene could not be assayed in gradient fractions, due to the high levels of phenolic compounds present in membrane fractions, particularly those from cotton and cucumber cotyledons.

Gradients fractionated following centrifugation of castor bean, cotton, cucumber and soybean cotyledon homogenates were routinely assayed for isocitrate lyase (glyoxysomal marker) and fumarase (mitochondrial matrix marker) to evaluate separation of glyoxysomes from mitochondria. The location of mitochondrial and glyoxysomal bands was invariant and consistent with what depicted in Fig. 1, provided that the seedlings used were more than 4 days old to avoid multiple mitochondrial bands (data not shown).

Transmission electron micrographs of sections from pellets obtained upon centrifugation of cotton cotyledon mitochondria appeared to contain intact mitochondria with remarkably few non-mitochondrial membrane vesicles. Some lower sections did contain some unidentified membranes but no glyoxysomes or intact plastids were visible (data not shown).

The data represented in Tab. 1 illustrate the range of

Tab. 1. Range of values obtained for activity and integrity parameters using mitochondria isolated by the abbreviated method. RC ratio refers to the respiratory control ratio. Integrity refers to the percentage of mitochondria with intact outer membranes (see Materials and methods). Numbers in parentheses by tissue source refer to number of measurements (from independent preparations) represented by range of values. Letters in parentheses indicate substrate used in indicated parameter: A, succinate; B, malate; C, α-ketoglutarate; ND, not determined. Yields of mitochondria are given as mg mitochondria (g fresh weight)⁻¹. State 3 rates of oxygen consumption are expressed as nmol O₂ (mg mitochondrial protein)⁻¹ min⁻¹.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Castor bean (3)</th>
<th>Cucumber (2)</th>
<th>Cotton (4)</th>
<th>Soybean (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield</td>
<td>0.27-0.38</td>
<td>0.30-0.33</td>
<td>0.10-0.16</td>
<td>0.12-0.23</td>
</tr>
<tr>
<td>State 3 rates</td>
<td>456-643 (A)</td>
<td>45-184 (B)</td>
<td>156-260 (A)</td>
<td>48-79 (B)</td>
</tr>
<tr>
<td>RC ratio</td>
<td>3.1-4.2 (A,B)</td>
<td>2.9-4.0 (A,C)</td>
<td>1.8-3.2 (A)</td>
<td>4.2-7.8 (A,C)</td>
</tr>
<tr>
<td>ADP:O ratio</td>
<td>2.29-2.59 (A)</td>
<td>1.94-2.38 (A)</td>
<td>ND</td>
<td>1.94-2.69 (A)</td>
</tr>
<tr>
<td>Integrity (%)</td>
<td>95-99</td>
<td>86-92</td>
<td>82-88</td>
<td>ND</td>
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References


