Mitochondrial Purification

Sample (8 g) was ground in 12 mL of grinding buffer containing 0.45 M mannitol, 50 mM sodium pyrophosphate, 2 mM EGTA, 0.5% polyvinylpyrrolidone-40, 0.5% bovine serum albumin, and 20 mM cysteine (pH 8.0). The homogenate was filtered through four layers of Miracloth (Calbiochem, Darmstadt, Germany) via a funnel into a beaker. The speed and yield of this process were increased by gently soaking the cloth in the funnel. For the optimum production of mitochondria, all processes were performed at 4 °C. The filtered homogenate was transferred into a 50 mL falcon tube, and then centrifuged at 1,500 × g for 5 min. The supernatant was gently removed without disturbing the pellet, which contained starch, nuclei, and other cell debris, and then the supernatant was transferred into a set of ultracentrifuge tubes. The supernatant (8 mL), which contained the crude organelles, was layered over a Percoll gradient prepared in a 50 mL ultracentrifuge tube; from top to bottom, the gradient consisted of 7 mL 40% Percoll, 10 mL 23% Percoll, and 7 mL 18% Percoll in mannitol wash buffer containing 0.3 M mannitol, 10 mM MOPS (pH 7.5), and 0.1% bovine serum albumin. The tube was centrifuged at 34,000 × g for 1 h at 4 °C with the brake turned off during deceleration. Mitochondria were visible as a series of dense bands at the 23%–40% Percoll interface. The mitochondrial layer was collected by using a Pasteur pipette, and transferred to a new ultracentrifuge tube. The samples were diluted with sucrose wash buffer containing 0.3 M sucrose and 10 mM MOPS (pH 7.5), and then centrifuged at 24,000 × g for 30 min at 4 °C twice. The supernatant was discarded. To remove the salt, lipids, chlorophyll, and other cellular components, nine times the sample volume of cold acetone was added to a new tube and placed at -20 °C overnight . The precipitated sample was centrifuged at 20,000 × g for 15 min at 4 °C. The supernatant was discarded and the pellet was dried for 15 min at room temperature. The pellet was suspended in SDS buffer containing 1 M Tris-HCl (pH 6.8), 50% glycerol, 20% SDS, and 0.5% 2-mercaptoethanol for Western blot analysis, or lysis buffer consisting of 7 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine for proteomic analysis.

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