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Abstract

Isolation of subcellular fractions

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Isolation of subcellular fractions of Neurospora mycelio.

The following procedure for the isolation of subcellular fractions from Neurospora mycelia has been developed in our laboratory during the post two years.

Cultures: Two 125 ml Erlenmeyer flasks containing 20 ml of Vogel's minimal medium supplemented with 1.5% sucrose and 1.5% agar ore inoculated, cultured first at 30°C for 3 days in the dark and then at mom temperature for 4 days with continuous illumination. Conidia are harvested in 50-100 ml of distilled water and transferred to a 5-gallon carboy containing 15 liters of sterile Vogel's minimal medium supplemented with 1.5% sucrose. After 3 days' incubation with continuous aeration at 25-30°, mycelio ore collected on a double layer of cheesecloth in a sieve 40 cm in diameter. The mycelio ore washed on the sieve with a freshly prepared solution of 0.50 M sucrose (Mann Co., Enzyme Grade) containing one mM dipotassium EDTA (Sucrose EDTA) at 4° and 50 mM Trls Cl, pH 7.4. The mot is passed through a hand clothes wringer and blotted with paper towels. The yield of wild-type (74A) is generally 200 grams moist weight.

Disruption of cells: Small pieces of mycelium ore placed in a 1-quart Waring blendor with sucrose-EDTA solution at 4°. About 7 ml of solution per gm fresh weight is sufficient. The mixture is blended for 6 seconds at the "high" speed of the blendor and poured into a Roalox mill jar (size No. 1) with 2 liters of glass beads. Prior to UK, the beads (Van Waters and Rogers Co., Cot. No. 3400708, I mm diameter) are thoroughly washed on a 20-mesh sieve with water and sterilized in on oven at 160° for at least 3 hours. The jar is rotated at 288 rpm with a jar mill for at least 1-1/2 hours at 4°.

The supernatant is poured from the jar and set aside. The beads ore washed at least 6 timer with a total (1 to 1.5 volumes) of sucrose-EDTA and the washes ore combined with the supernatant.

Isolation of subcellular fractions: Large pieces of unbroken mycelium and residual beads ore removed from the homogenate by vacuum filtmtion through a single layer of double-napped pajama flannelette on a 30-cm Buchner funnel. The filtrate, designated as H_1, is centrifuged at 2,000 x g for IO minutes in the VRA rotor of a Lourdes Betafuge. The crude nuclear precipitote (N) is set aside (N1) the cold and the supernatant is centrifuged at 16,000 x g for 20 minutes. The mitochondrial precipit a t is set aside (M1) the cold and the supernatant is again centrifuged at 16,000 x g for 20 minutes. The slight precipitate is com ined the M_1 mb100ml of sucrose-EDTA. The supernatant is then centrifuged at 120,000 x g for 2 hours in the No. 50 rotor of a Spinco Model L-2 ultracentrifuge. The microsomal precipitate (P) is suspended in 5-10 ml of sucrose-EDTA with the oid of a glass-teflon homogenizer.

Additional purification of nuclear and mitochondrial fractions: Fraction N_1 is suspended in 10-12 ml of a solution containing 0.5 M sucrose and 10 mM $CaCl_2$ and filtered through double-napped flannel&e. The filtrate is centrifuged at 500 x g for 2 minutes and the fluffy layer of nuclei, together with the supernatant, is decanted from the hard pellet of cell-wall debris and unbroken cells. law-speed centrifugation is repeated twice. The crude nuclear fraction (5-10 ml) is layered on 5 ml of 2.3 M sucrose with a syringe ond centrifuged at 5,000 x g for 15 minutes. Nuclei are withdrawn from the density shelf and layered with a syringe on 5 ml of 1.5 M sucrose containing IO mM $CaCl_2$. After centrifugation at 20,000 x g for 20 minutes, the supernatant is withdrawn from the tube with a syringe and discarded. The nuclear pellet (N2) remains at the bottom.

Additional purification of the crude mitochondrial fraction (M_1) is obtained by a "double-shelf" technique. 1.2 ml of 1.2 M sucrose is layered upon 5 ml of 1.5 M sucrose. 5 ml of a suspension of crude mitochondria (in 0.5 M sucrose) ore layered above the double layers. After centrifugation at 16,000 x g for 30 minutes, mitochondria are removed from the central layer with a syringe fitted with a wide-gauge, blunt-tipped cannula. The suspension is diluted to 0.5 M sucrose and the double-shelf centrifugation is repeated.

Table 1 shows the distribution of total protein in the various subcellular fractions.

Discussion: Probably no one procedure con be devised that is ideal for the isolation of all of the various subcellular organelles from one cellular homogenate. The procedure described is therefore a compromise. For example, $CaCl_2$ is necessary for stability of nuclei, (Reich and Tsuda 1961 Biochim. Biophyr. Acto 53: 574). However, mitochondria tend to aggregate in the presence of divalent cations. Similarly, the preservation of ribosomes with Mg^{++} leads to the sedimentation of aggregated mitochondrio with the nuclear fraction.

Table 1. Distribution of total protein in subcellular fractions of Neurospora mycelia.

Fraction	Centrifuge force (x 1,000 g)	Time (minutes)	Protein (mg) recovered in fraction per 100 mg total Protein *
N_2	2	10	10 ± 6
M_2	lb	20	28 ± 12
P	120	120	15 ± 7
S	su		49 ± 13
		Total	100**

* Average and standard deviation of four experiments with wild-type (74A). Protein was measured by a biuret method after samples were dissolved in 8 M urea containing 0. 1 N NaOH.

** Average recovery in the sum of the fractions was 92% of the protein in the filtered homogenate (H_1). About 3 gm protein is obtained in H_1 Per 200 gm fresh mycelium.

The fine structure of mitochondria prepared by this method is indistinguishable from mitochondria in hyphoe in electron micrographs of the sections stained with osmium tetraoxide and uranyl acetate. Nuclear preparations, although apparently free of mitochondria, are variable in size and shape, and contain some ruptured nuclei.

Systematic ond empirical variation of the conditions for cell disruption ond fractionation may be necessary in order to study enzyme localization. Malate dehydrogenase is found exclusively associated with mitochondria in situ by histochemical methods ond electron microscopy. On the other hand, the recovery of malate dehydrogenase in the mitochondrial fraction is a direct and linear function of the sucrose concentration of fhe isolation medium. - - - Deportment of Biological Sciences, Stanford University, Stanford, California. 94305.