The Isolation of Nuclei from the Filamentous Fungus *Aspergillus nidulans*

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(Received 4 August 1975; revised 25 November 1975)

SUMMARY

A method has been developed for isolating nuclei from the filamentous fungus *Aspergillus nidulans*. In this procedure, the mycelia from 14 to 16 h spore-derived cultures of *A. nidulans* NRI, a stable diploid strain, were frozen with liquid nitrogen and homogenized in a Waring blender. After homogenization, the mycelia were warmed to 4 °C and the nuclei were purified from the homogenate by differential centrifugation followed by sedimentation through 2.1 M-sucrose. The final nuclear yield was 15 to 20 %, based on DNA estimations. The purified nuclear pellet was free of whole cells. The morphology of the isolated nuclei resembled that of the *in situ* nuclei; they contained a nucleolus, chromatin, and had a surrounding double membrane. The purified nuclei were characterized by a DNA:RNA:protein ratio of 1:2.8:8.7.

INTRODUCTION

It is difficult to isolate and purify the nuclei of filamentous fungi in the amounts necessary for biochemical analysis. The major problem encountered is to disrupt the tough cell wall without simultaneously destroying the fragile nucleus. Enzymic digestion of the cell wall has been used to form protoplasts of *Saccharomyces* species which can then be lysed to release nuclei (Doi & Doi, 1974; Rozijn & Tonino, 1964; Wintersberger, Smith & Letnansky, 1973). Although protoplast formation using *Aspergillus nidulans* has been reported (Peberdy & Gibson, 1971) we have been unable to establish conditions under which it is possible to process sufficient quantities of mycelia to obtain nuclei for biochemical analysis. Physical disruption of the cell wall has been used to isolate nuclei from *Saccharomyces cerevisiae* (Bhargava & Halvorson, 1971), *Achyla bisexualis* (Jaworski & Horgen, 1973), and *Neurospora crassa* (Reich & Tsuda, 1961). Freezing of mycelium in liquid nitrogen before homogenization has been used to isolate *N. crassa* mitochondria (Minssen & Munkres, 1973) and polyribosomes (Sargent, 1973).

We report here the details of an isolation procedure for *A. nidulans* nuclei based on homogenization in liquid nitrogen. The criteria used for this nuclear purification were: the absence of any intact cells in the final nuclear fraction; the morphological similarity of *in situ* and isolated nuclei; and the presence of nuclear proteins, e.g. histones. The characterization of histones from the purified nuclei has been reported elsewhere (Felden, Sanders & Morris, 1976).

METHODS

Organism. *Aspergillus nidulans* (Eidam) Wint. strain NRI was used; it is a stable diploid strain which is homozygous for a temperature-sensitive mutation at the *bim-7* locus. At the restrictive temperature, this strain exhibits an increased mitotic index and inhibition of

nuclear division. The diploid strain was the result of a mating between UVTS706, the original *bim-7* isolate from FGSC154 (Morris, 1976), and a derivative of FGSC99 into which the same *bim-7* conditional mutation had been crossed. The genotype of UVTS706 is *ade-20, bio-1, whi-2, nit-3, slp12, met-1, nic-2, lac-1, bim-7, cho, cha*. The genotype of the *bim-7* FGSC99 strain is *sup-1 ade-20, pro-1, pab-1, ylo, ade-20, Acr-1, nic-2, lac-1, bim-7*. The resulting diploid required nicotine, would not utilize lactate, had a leaky adenine requirement, and was more sensitive to acriflavin than the wild type. A diploid strain was used to develop this nuclear isolation procedure because its increased nuclear size and mass aided purification.

Growth. Cultures were grown for 14 to 16 h at 32 °C with shaking (200 rev./min) in YG medium [0.5 % (w/v) yeast extract, 2 % (w/v) dextrose]. An initial inoculum of 1×10^8 to 5×10^8 conidia/ml in 1 l of medium gave a final yield of approximately 10 g blotted wet weight.

Microscopy. Nuclear isolation was monitored by fluorescence microscopy using a Zeiss Universal microscope with vertical illumination (exciter filters BG-38 and BG-12, barrier filter 53/44). Acridine orange (C.I. 46005) at a concentration of 50 mg l⁻¹ was used to stain the material; the same concentration was used by Clutterbuck & Roper (1966). Homogenization buffer was used as the solvent for the stain because its osmotic properties helped preserve the nuclear structure. Before staining, whole cells were fixed in modified Helly solution as described by Robinow & Caten (1969); but the homogenate and purified fractions were stained without fixing so that nuclear purification could be evaluated without causing any delay in the isolation procedure. With fluorescence microscopy, the nuclei were easily and rapidly distinguished from other cellular components, many of which, after homogenization, were indistinguishable by either light or phase-contrast microscopy.

For electron microscopy, the material was fixed in 1.5 % (w/v) *p*-formaldehyde and 1 % (v/v) glutaraldehyde in the homogenization buffer, post-fixed with 1 % (w/v) osmium tetroxide in veronal acetate buffer (pH 6.2), stained with 0.5 % (w/v) uranyl acetate in the same buffer, and post-stained with lead citrate. Sections were observed at 80 kV with a JEM 100C electron microscope.

Solutions. The homogenization buffer contained: 10 mM-PIPES (piperazine-*N,N'*-bis-2-ethane sulphonic acid, Sigma P-6757; pH 6.9); 5 mM-CaCl₂; 5 mM-MgSO₄ and 0.5 M-sucrose. The buffered 2.1 M sucrose solution was identical to the homogenization buffer except that 2.1 M-sucrose was substituted for 0.5 M-sucrose.

DNA, RNA and protein analysis. All treatments were performed at 4 °C except where noted. Samples to be analysed were precipitated with perchloric acid (PCA, final concentration 0.25 M) for 30 min. The precipitate was centrifuged, resuspended in distilled water, and sonicated (Sonifier cell disrupter, Model W185D, Bronson Power Co., Plainview, N.Y., U.S.A.) using two 15 s bursts at 55 W with the micro-tip. The sonicate was washed (10 to 20 vol./wash) twice with 0.5 M-PCA, once with 95 % ethanol, and once again with 0.5 M-PCA. Nucleic acids were extracted twice for 20 min with 3.5 ml of 0.5 M-PCA at 85 °C. Protein was dissolved in 1 M-NaOH at 60 °C for 1 h.

DNA was determined by the diphenylamine reaction (Giles & Myers, 1965) and RNA by the orcinol reaction (Schneider, 1957). Protein was determined by the method of Lowry *et al.* (1951).

Isolation of nuclei. The nuclear isolation procedure is outlined in Fig. 1. Shake cultures were collected by filtering through Miracloth (Chicopee Mills Inc., New York, U.S.A.; Calbiochem 475855), washed with deionized water at room temperature, blotted dry and weighed. Each 150 g of blotted mycelium was resuspended in approximately 100 ml of homogenization buffer and mixed to a paste-like consistency. This mixture was then spooned

Fig. 1. A schematic diagram of the procedure used to isolate nuclei from *A. nidulans*. Details are given in the Methods.

into liquid nitrogen (about 2 l). Less than 15 min was required to collect, blot dry, resuspend and freeze 60 to 120 g mycelium. The frozen hyphae were homogenized in a Waring blender (Model CB-5) set at highest speed; blade speed was controlled by a powerstat variable autotransformer (Superior Electric Co., Bristol, Connecticut, U.S.A., type 3PN1168). Five 15 s bursts with the powerstat set at 60 to 70 V were used to grind 30 g mycelium. Approximately 250 ml liquid nitrogen was added between grinding bursts. At the same time, the powerstat was set at 40 V to prevent the blade mechanism from freezing. The homogenized mycelium had the consistency of fine beach sand. By light microscopy it was estimated that 25 to 50 % of the hyphae were broken. The homogenate was rapidly warmed to 4 °C by placing it in an 80 °C water bath; all subsequent manipulations and centrifugations were done at 4 °C. After adding 50 to 100 ml of homogenization buffer, a motor-driven Teflon-glass tissue grinder (Inframo motor, model RZR64; setting 5) was used to extrude nuclei sequestered within hyphae. The resulting homogenate was diluted with four volumes of homogenization buffer and filtered through Miracloth, a fresh filter being used for each 100 ml of diluted homogenate. It was necessary to dilute the homogenate and to change the filter in order to prevent occlusion of free nuclei by the hyphal mat. The filtrate was centri-

fuged at 6000 *g* for 20 min to sediment the nuclei. The resulting pellet (P₁) was resuspended in approximately 150 ml of homogenization buffer and centrifuged at 100 *g* for 5 min to remove any large hyphal pieces which had passed through the Miracloth. The supernatant (S₂) was removed and the nuclei were again sedimented by centrifuging at 6000 *g* for 20 min. The resulting nuclear pellet (P₃) was resuspended in the 2.1 M-sucrose buffer and centrifuged at 27000 *g* for 5 min to remove small mycelial fragments and any remaining whole cells. The resulting supernatant (S₄) was centrifuged at 46000 rev./min in a Spinco type 50 rotor (161000 *g*) for 50 min, and yielded a purified nuclear pellet (P₅). Mitochondria and membranes floated to the top of the tube and formed a pellicle in this final centrifugation. The purified nuclear pellet was free of any whole cells.

RESULTS AND DISCUSSION

Nuclei in situ

The nuclei of *A. nidulans* were observed by fluorescence microscopy after fixing with modified Helly solution. When *A. nidulans* NR1 was grown at 32 °C, the nuclei appeared as evenly spaced, brightly stained, green ovoid bodies (Fig. 2*a*). Within each nucleus, the nucleolus appeared as a small red spherical region. The electron microscope revealed the nuclear structure in more detail (Fig. 2*b*). The diploid nucleus at interphase measured approximately 1.8 µm in diameter. The chromatin had both dark and light stained areas, presumably heterochromatin and euchromatin. Each nucleus was enclosed within a double membrane.

Nuclear isolation

Nuclei were isolated from 14 to 16 h spore-derived cultures as described in Methods. Freezing the mycelia in liquid nitrogen had two advantages: (i) it increased mycelial breakage during homogenization; and (ii) it would be expected to stop enzymic reactions instantaneously and, therefore, minimize degradation of nuclear components. Other methods of cell disruption were less successful. Homogenization of unfrozen mycelium in a Waring blender, even after incubation with snail gut enzyme, resulted in less cell breakage. Methods which caused massive cell disruption, such as the French press, also resulted in the destruction of practically all nuclei. Attempts to form protoplasts of *A. nidulans* on a scale large enough to isolate nuclei for biochemical analysis were unsuccessful. Apparently, liquid nitrogen freezing makes the cell sufficiently brittle to be sheared yet ensures sufficient protection of nuclei to prevent disruption.

The nuclear isolation procedure was monitored by fluorescence microscopy of acridine orange stained samples, which allowed us to follow the purification and determine the integrity of the nuclei. Under our conditions, the nucleus appeared as a green sphere containing a smaller red sphere; this distinct nuclear unit was readily distinguishable from all other cell constituents in the same fraction. Attempts to distinguish nuclei from other particles in the homogenate or partially-purified fractions were unsuccessful when either light or phase-contrast microscopy was used. Fluorescence microscopy also allowed us to detect nuclear disruption by looking for the separation of the red nucleolus from the rest of the nucleus and/or for leakage of the green chromatin from the nucleus. Inspection of the purified nuclear fraction showed that the chromatin was contained within the nuclear structure and that the nucleoli were still attached in approximately 70 % of the nuclei (Fig. 3). Electron micrographs showed that isolated nuclei were structurally similar to the nuclei *in situ* (Fig. 4). A double nuclear membrane could be seen attached to the nucleus. The nuclear membrane was not complete in all nuclei and this may have been a direct result

Fig. 2. *In situ* nuclei of *A. nidulans* NR1. (a) Fluorescence micrograph of hyphae fixed in modified Helly solution and stained with acridine orange. (b) Electron micrograph of a section through a nucleus, showing chromatin (C), nucleolus (Nu) and double nuclear membrane (M).

Fig. 3

Fig. 4

Fig. 3. Isolated nuclei from *A. nidulans* NR1. Nuclei from the final purified fraction P5 (see Fig. 1) were stained with acridine orange without prior fixing.

Fig. 4. An isolated nucleus from *A. nidulans* NR1. The electron micrograph shows chromatin (C), nucleolus (Nu) and double nuclear membrane (M).

of our procedure. Membrane connexions between the nucleus and endoplasmic reticulum have been described in other fungi (Bracker, 1967) and it is also possible that breakage of such a connexion during homogenization resulted in the rupture of the nuclear membrane. Nuclei which lacked part of their envelope were, nevertheless, not disrupted. The morphology of the chromatin and nucleolar material of the isolated nuclei were similar to the *in situ* nuclei. The nucleolus appeared densely packed with small, darkly-stained spheres while the chromatin showed dark and light regions, presumably heterochromatin and euchromatin, respectively. Contaminating material in the purified nuclear pellet consisted of membrane-bound ribosomes, plasma membrane and pieces of cell wall.

Some 15 to 20 % of the cellular DNA was recovered in the purified nuclear pellet (Table 1). The major loss of DNA occurred during the filtration to remove unbroken hyphae after

Table 1. *Composition of Aspergillus nidulans nuclei**

Fraction	Percentage yield of DNA	Relative composition		
		DNA	: RNA	: Protein
Homogenate	100	1	: 40.8	: 89.2
P1	35.7	1	: 14.7	: 35.2
P3	19.5	1	: 6.2	: 23.7
P5 (nuclear fraction)	18.8	1	: 2.8	: 8.7

* Average of two experiments.

Table 2. *Composition of isolated nuclei*

	Percentage yield of DNA	Relative composition		
		DNA	: RNA	: Protein
<i>Aspergillus nidulans</i>	18.8	1	: 2.8	: 8.7
<i>Saccharomyces cerevisiae</i>				
Doi & Doi (1974)	24	1	: 2.7	: 13.7
Wintersberger <i>et al.</i> (1973)	36	1	: 4.2	: 17.3
Bhargava & Halvorson (1971)	46.7	1	: 2.5	: 15.6
<i>Neurospora crassa</i> (slime)				
Hsiang & Cole (1973)	46	1	: 6.3	: ND
Rat (Tata, 1974)				
Liver	70.4	1	: 0.20	: 3.7
Heart	43.0	1	: 0.26	: 3.9

ND, not determined.

homogenization. The first nuclear pellet (P1) contained only about 35 % of the DNA content of the homogenate. Approximately 50 % of the DNA in P1 was recovered in the final nuclear fraction (P5). An attempt to increase the yield of nuclei with the non-ionic detergent Triton X-100 was not successful at concentrations of 0.1 or 0.5 % (v/v). Fluorescence microscopy revealed that at the higher detergent concentration the nuclei appeared more disrupted, and electron microscopy showed that the nuclear membranes were removed from most nuclei. The overall recovery of DNA from *A. nidulans* was less than reported for *S. cerevisiae* (Bhargava & Halvorson, 1971; Doi & Doi, 1974; Wintersberger *et al.*, 1973) or for the *N. crassa* slime mutant (Hsiang & Cole, 1973). However, our recovery was better than that of Hsiang & Cole (1973), who obtained a recovery of less than 10 % from wild-type *N. crassa* using the disruption method of Reich & Tsuda (1961). The low nuclear yield is partly compensated for by the fact that *A. nidulans* grows rapidly and that our method allows us to handle large amounts of mycelium. We are therefore able to isolate nuclei in sufficient amounts for biochemical analysis and characterization of components.

Table 1 also shows that the RNA:DNA and protein:DNA ratios decreased approximately 10-fold during purification. Since DNA, RNA and protein are all nuclear components, we cannot determine with certainty the degree of purity of the nuclei, but the 10-fold decrease in the ratios suggests at least a 10-fold nuclear enrichment. Table 2 compares the macromolecular composition of *A. nidulans* nuclei to that of nuclei from other organisms. The fungal nuclei appear to have a high content of RNA relative to DNA, compared with mammalian nuclei. This may be due to contamination with ribosomal RNA. However, *A. nidulans* has a larger nucleolus than those found in mammalian nuclei; we therefore suggest that the high nuclear RNA value may reflect the RNA content of the large nucleolus.

The authors wish to thank Dr B. Ghosh (Rutgers Medical School) for his aid in the preparation of electron micrographs and G. Wang and M. Lai for their technical assistance. This research was supported by grants CA 10665 of the U.S. Public Health Service and GB 43404 of the National Science Foundation.

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