Nuclear density determination and the purification of wild type Neurospora nuclei using Percoll gradients.

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Abstract
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We compared the sensitivity of this procedure to two other common protease assays using hemoglobin (Kunitz 1947 Proc. Soc. Exp. Biol. Med. 30:291) and casein yellow (Anson 1938 J. Gen. Physiol. 22:79). The latter assays are short-term; so we modified our procedure to include a reaction termination step, adding 0.5 ml of 10% trichloroacetic acid at appropriate time intervals. We found that azocoll is approximately 100-fold more sensitive, over a 30 min interval, to hydrolysis by trypsin than are either casein yellow or hemoglobin (Figure 3). To demonstrate further the sensitivity of the azocoll assay, we assayed several commercial proteases over a ten h. period and readily detected one nanogram quantities of trypsin, subtilisin, and thermolysin (Sigma).

The solid reagent dispenser greatly reduces the time needed to measure azocoll for each reaction mixture and makes feasible the use of azocoll for investigations requiring large numbers of protease assays. This method of measuring azocoll is both rapid and accurate (machine error is ±4%). The dispenser could readily be used to measure other insoluble substrates. Moreover, by using teflon rods with suitably sized holes drilled in them, one could dispense different amounts of solid substrates. (This research was sponsored jointly by NSF Grant PCM 76-80227 and the Office of Health and Environmental Research, U.S. Dept. Energy; Contract W-7405-eng-26 with Union Carbide.)

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The study of precursor ribosomal RNA (pre-rRNA) maturation in ribosome biosynthesis mutants of Neurospora crassa is facilitated by the isolation of RNA from purified nuclei. Problems have been encountered in attempts to purify nuclei with Luddox gradients. Specifically, Luddox precipitates at low temperatures when exposed to Triton X-100, which is an essential component of the buffer used in the nuclei isolation steps. Therefore, a new gradient medium Percoll (Pharmacia Fine Chemicals, Piscataway, N.J.) was tested for its applicability. The use of Percoll rather than Luddox eliminated problems with precipitation. In addition it was possible to determine the buoyant density of the nuclei accurately, since the colloidal silica particles are coated with polyvinylpyrrolidone to which the nuclear membrane is impermeable.

Flasks of liquid Vogel's minimal medium inoculated with wild type conidia (2x10^7 ml^{-1}-1) were incubated for 8 h. at 25°C. Crude nuclear pellets were prepared from these mid-logarithmic phase cultures using a modified version of the procedure described by Hautala et al. (1977) J. Bacteriol. 130:704). As in the original method, a French pressure cell was used for efficient cell breakage. Modifications included centrifuge of the supernatant liquid from the post-Omnimixer homogenized cell suspension at 2,300 g (rpm 8.26 cm) rather than 530 g for each centrifugation. For subsequent steps, changes in buffer B were necessary to maintain correct ionic strength for the Percoll gradient step. To generate a medium having an osmolality of 320 mOsm/kg H_2O, it is necessary to mix Percoll with 2.5 M sucrose in a 9:1 ratio. Lower starting densities of Percoll can be obtained by adding the appropriate amount of 0.25 M sucrose. Since, in the Hautala method, the crude nuclear pellet is suspended in buffer B which contains 1 M sucrose (i.e., 50 mM Tris-HCl, pH 7.5; 5 mM MgCl_2; 10 mM CaCl_2; 1 M sucrose; and 1% (v/v) Triton X-100), it was necessary to reduce the sucrose concentration in the experiments reported here from 1.0 to 0.25 M while keeping the other ingredients the same.

Thus, the crude nuclear pellets that were obtained were suspended in 8-10 ml of the modified buffer B and homogenized in 40-ml Potter-Elvehjem tissue grinders. The suspensions were then mixed with the appropriate amount of Percoll (isotonic in 2.5 M sucrose) in Beckman 1.6 x 7.62 cm 10.4 ml polycarbonate bottle assemblies.
which were centrifuged at 4 C for 45 min at 58,300 g (w~6.66 cm) using a DuPont-Sorvall OTD-2 ultracentrifuge. Owing to the size heterogeneity among Percoll particles, they sediment (and diffuse) at different rates in a gravitational field, thereby creating a density gradient. The biological material in the gradient, in this case nuclei, bands isopycnically, so that the sample particles reach a position where their densities and that of the surrounding Percoll medium are equal. As is the case with isopycnic separation using cesium chloride gradients, a fixed angle rotor has advantage over a swinging bucket rotor since with fixed angle rotors reorientation of the tube contents does not occur to alter the final separation of the zones and there is better resolution of the experimental materials since they are banded over a larger cross sectional area.

A range of starting densities of Percoll from 1.05 to 1.12 gm$^{-1}$ were tested in separate experiments to determine the most useful for banding Neurospora nuclei. After each experiment the nuclei were fractionated into 12 fractions, and their refractive index determined with an A/O Refractometer. The results showed that the centrifugation generated adequate Percoll gradients. The nuclei banded to one region of the gradient but the band was not homogeneous: the upper part was relatively disperse, the center was dense and homogeneous, and the lower part exhibited some clumps. Based on refractive index measurements, the density of the nuclei was determined to be 1.078 gm$^{-1}$. The nuclei may be recovered by centrifuging gradient fractions containing nuclei for 2 h at 100,000 g (w~) in a swinging bucket rotor. Under these conditions, the silica particles pellet and the nuclei remain above the gel formed. The nuclei may then be pelleted from the supernatant liquid by centrifugation for 20 min at 5,000 g (w~).

In conclusion, the results indicate that Percoll is an effective alternative to Ludox for the purification of Neurospora nuclei from crude nuclear preparations. The absence of large osmotic effects such as is observed with other gradient materials has allowed the density of wild type nuclei to be determined. Finally, although RNA extracted from crude nuclei includes high molecular weight species that are presumptive precursors to mature rRNA (K. Talbot 1980 Baccalaurate Thesis, Reed College), studies of pre-rRNA processing in the nuclei will be greatly facilitated now that pure nuclei can be obtained. (Supported by NIGMS, NIH grant GM22488). - - - Biology Department, Reed College, Portland, Oregon 97202.

Wootton, J. C., M. J. Fraser, and A. J. Baron. Efficient transformation of germinating Neurospora conidia using total nuclear DNA fragments.

We have developed a simple, reliable transformation method that does not require enzymic digestion of cell walls. This method exploits the tendency of \textit{inl} and \textit{os} strains to form swellings resembling sphaeroplasts on germ tubes when macroconidia are germinated in the absence of inositol in media of high osmolarity. Prototrophic transformants have been obtained from several auxotrophs of low or zero spontaneous reversion frequency, including the well defined deletion strain \textit{am 15}. Donor DNA was prepared from nuclear pellets by essentially the method of Hautala et al. (1977 J. Bacteriol. 130:704) and was usually used as linear fragments of average molecular weight at 2 x 10$^7$ without further shearing. DNA sheared to an average molecular weight of 5 x 10$^4$ gave similar results. Restriction fragments and single stranded DNA (the latter obtained by melting and rapid cooling of unprepared preparations of nuclear DNA as above) have also been used successfully. Detailed results will be reported elsewhere (submitted for publication).

In a typical experiment, 2 x 10$^8$ conidia of the recipient strain were germinated in 1 ml of Vogel's minimal medium containing 20% sucrose (w/v) and all required supplements except inositol for 3 to 5 hours at 30$^\circ$C on an orbital shaker. When the majority of germ tubes showed swellings indicating weakened cell walls, the conidia were harvested by centrifugation, washed 3 times with Vogel's minimal liquid medium containing 1M-mannitol and suspended in 0.4 ml of this medium. Donor DNA (1 to 5 \textmu}g) was precycled by mixing with 0.1 ml of 500 mM-CaCl$_2$ and added to the suspension of germinated conidia. Incubation was continued for 1 h at 30 C on an orbital shaker. Treated suspensions were diluted in 0.8M-mannitol for plating (either by spreading or in thin layers of soft agar) on sorbose medium containing 0.6M-mannitol and supplements as appropriate for selection of prototrophs or viability measurements. Viability of partially sphaeroplasted germinated conidia obtained by this method is generally greater than 90% Control treatments were also included using DNA prepared from the recipient, DNase-digested DNA, and CaCl$_2$ alone.

Transformation frequencies obtained by this method using \textit{inl} recipients ranged from 0.5 to 12.3 transformants per \textmu}g of DNA (1.5 x 10$^{-7}$ to 4.2 x 10$^{-6}$ per viable recipient conidium), with a mode around 5 transformants per \textmu}g of DNA (1.5 x 10$^{-6}$ per viable conidium). \textit{os} recipients have given similar results. For comparisons, the same recipients have been used in transformations employing the mycelial fragment method described by Mshra and Tatum (1973, Proc. Natl. Acad. Sci. 70:3875) and a protoplasting method similar to that of Hinnen et al. (1978 Proc. Natl. Acad. Sci. 75:1929). Both of these methods gave transformation frequencies in the 0.04 to 0.12 transformants per \textmu}g of DNA (0.8 x 10$^{-7}$ to 2.9 x 10$^{-7}$ per viable fragment or protoplast plated), approximately 50-fold lower frequencies than those obtained by our method using germinating conidia.

The choice of \textit{os} strains may be important. Best results have been obtained with recipients carrying \textit{inl} 37401 (FGSC 2145) and \textit{inl} R233 crossed from an isolate in our collection originally obtained from R. Gross. However, the properties of \textit{inl} R233 stocks changed after 3 backcrosses into STA4 background, and very low transformation frequencies were subsequently obtained. Presumably the kinetics of the lytic processes involved in inositol-less death are important for successful partial sphaeroplasting and may be influenced by genetic background... Department of Genetics, University of Leeds, Leeds LS2 9JT, United Kingdom.