NOTES

Analysis of Dictyostelium mucoroides Macrocysts by Using a Simple Breakage and Fractionation Procedure

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A method is described for the breakage and fractionation of Dictyostelium macrocysts. It involves vortexing with large glass beads to disrupt the macrocyst wall followed by differential low-speed centrifugation. It yields fractions containing purified wall material and intact endocytes. The endocytes can themselves be disrupted by using smaller glass beads.

In cellular slime molds the formation of macrocysts forms part of the sexual cycle and represents an alternative developmental pathway to fruiting-body formation (10). The macrocysts develop within an aggregate of thousands of myxamoebae. Two cells (of differing mating type in heterothallic strains) fuse to form a giant cell (or cytophagic cell) which progressively engulfs all of the surrounding cells (2, 3). These engulfed cells, known as endocytes, then disappear in a process apparently similar to digestion of food in vacuoles of vegetative myxamoebae (3). Although detailed ultrastructural studies have been made (2, 3), relatively little is known about the process of macrocyst formation at the biochemical and molecular levels. The macrocyst is a heavy-walled structure, and difficulty in breaking the trilaminate wall may have precluded studies concerned with the characterization and localization of macrocyst components.

In previous studies macrocyst disruption has been attempted by using sonication (12) or treatment with hydrolytic enzymes such as cellulase, pectinase, and α-amylase (4). Neither of these methods is entirely satisfactory and a simpler, more efficient method was clearly desirable.

By using a method based on procedures developed for disrupting bacterial (11) and fungal (6, 14) cells, spores of the cellular slime molds can be broken by vortexing with small (0.25- to 0.32-mm)-diameter glass beads (8, 13). We tried the same procedure with macrocysts, but the small beads proved to be of limited use. At early stages of macrocyst formation (up to the time of formation of the tertiary wall, 4-day cultures), some breakage was achieved, but excessive vortexing was required (20 15-s periods). As the macrocysts matured, they became even more resistant to breakage. Macrocysts have diameters of between 25 and 50 μm, an order of magnitude larger than spores. As there was likely to be a direct relationship between the sizes of the structures to be broken and the beads required, larger beads were tried. This not only proved to give good breakage, but also provided extracts which could be separated into fractions suitable for characterization of the component parts of the developing macrocyst.

Dictyostelium mucoroides DM7 (ATCC 42609) was grown in association with Escherichia coli B/r on 0.1% LP agar and harvested as described elsewhere (9). Pellets of macrocysts (three to six plates) were stored frozen until required, although freezing was not a prerequisite for successful breakage and fractionation. The standard procedure was usually carried out in glass tubes (10 by 1 cm) as follows. To each pellet were added 0.3 ml of acid-washed large glass beads (1.00- to 1.05-mm diameter, obtained from J. L. Van Etten, University of Nebraska, Lincoln; or 1.5- to 2.0-mm diameter, obtained from BDH) and sufficient extractions buffer (10 mM potassium phosphate, pH 6.5, containing 0.1% Triton X-100) to cover the beads to a depth of 1 mm (approximately 0.35 ml). With the tube held vertically, the sample was vortexed at between 1,000 and 2,000 oscillations per min for eight 15-s periods. The beads were allowed to settle and the crude macrocyst extract (fraction I) was removed with a Pasteur pipette. This was then centrifuged in a bench centrifuge (1 min, 1,480 × g) to give a pellet (fraction II) and supernatant (fraction III). Fraction II, which consisted of macrocyst wall and endocytes, was washed twice in 1 ml of extraction buffer and collected by centrifugation (1 min, 1,480 × g). Fraction III was separated into particulate (fraction IV) and soluble (fraction V) components by centrifugation in a microcentrifuge (1 min, 13,400 × g). Fraction IV was washed twice with 1 ml of extraction buffer and collected by centrifugation. The components of fraction II were separated further by a brief, low-speed centrifugation (5 s, 840 × g) which gave a supernatant (fraction IIA), enriched in endocytes, and a pellet (fraction IIB), enriched in macrocyst walls. Fraction IIA was centrifuged for 1 min at 1,480 × g, and the pelleted material was washed twice with 1 ml of buffer and collected by centrifugation. The pelleted endocytes were resuspended in a small volume of extraction buffer (0.10 to 0.30 ml), and acid-washed small glass beads (0.25 to 0.32 mm in diameter) were added to leave a 1-mm head of buffer. The sample was vortexed for 8 15-s periods to break the endocytes. The wall preparation (fraction IIB) was washed three times with 1 ml of extraction buffer, using two brief centrifugations (5 s, 840 × g) and one longer spin (1 min, 1,480 × g) to collect a firm pellet. All pelleted material

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was resuspended in extraction buffer before analysis by phase-contrast microscopy.

The effectiveness of the breakage and fractionation procedure is illustrated in Fig. 1. Rupture of a macrocyst usually resulted from a single split in the wall (Fig. 1B). Large glass beads split macrocysts of all ages (cultures up to 32 days old were examined). A high percentage of breakage could be maintained throughout development by increasing the vortexing time for older macrocysts, which were slightly more resistant. There appeared to be a critical minimum bead size, however, as 0.70- to 1.18-mm-diameter beads (Sigma), which were presumably of a smaller average size, gave poor breakage.

At early stages of development intact endocytes were released from the macrocysts (Fig. 1B and D). The original buffer used contained 0.1% Triton X-100, although identical results were obtained when the detergent was omitted. It was, however, surprising to find intact endocytes under these conditions. Even the inclusion of 1.0% (vol/vol) Triton X-100 during or after breakage failed to disrupt them. As endocytes are derived from ingested myxamoebae, it had been assumed that they would be surrounded by a membrane which, like that of myxamoebae, would be dissolved by Triton X-100. That this was not so implies that the membrane had been subjected to considerable modification or that the endocytes were walled. Rupture of the endocytes occurred after homogenization with small glass beads, with single splits appearing in the endocyte envelope (Fig. 1E). This effect was similar to that achieved with the same size beads on cellular slime mold spores or microcysts, both of which do possess walls.

Endocyte morphology changed as macrocysts developed. Initially they were irregular, slightly refractile bodies (Fig. 1D). As development proceeded, they became more refractile, denser, and smaller, and once mature macrocysts had formed endocytes could no longer be seen in the initial extract (fraction I). In 16-day cultures the endocyte-rich fraction (fraction IIA) contained small membranelike fragments, but no whole cells. The time course of the loss of endocytes from the cell preparations correlated with their gradual disappearance from the intact macrocysts observed here and previously (3, 4, 7).

The pelleted wall-rich fraction (fraction IIB) contained a matrix of laminated material (Fig. 1C). Some endocytes remained trapped within the walls, but the bulk of the material appeared to have been derived from the macrocyst wall.

After removal of the endocytes and walls by low-speed centrifugation, preparations from all stages of macrocyst development contained particulate material which could be pelleted by microcentrifugation. When examined by phase-contrast microscopy, the particles resembled small membrane fragments (not shown).

Most of the protein in the initial fraction I was soluble. Typically, none of the endocyte-rich (IIA), wall-rich (IIB), and particulate (IV) fractions contained more than 3% of the total protein recovered as determined by the Bradford (1) method; the remainder (>$90\%$ of the total) was found in the supernatant fraction V. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis using the standard Laemmli method (5) in a Bio-Rad mini-Protein system showed that there was some overlap between the protein components of the different fractions (Fig. 2). It is likely that this resulted partially from contamination of one fraction with another and should be eliminated when further refinements are introduced in the fractionation procedure. There were, nevertheless, some significant differences in the protein patterns. The wall-rich fraction prepared from younger macrocysts (4-day cultures) included five major...
proteins with apparent \( M_r \)s of 17,000, 28,000, 33,000, 39,000, and 44,000. One of these (28,000 \( M_r \)) was absent from vegetative cells, while the others were present in smaller proportions in myxamoebae than in the wall fraction. Two of the proteins (39,000 and 44,000 \( M_r \)) were also major proteins in the endocyte-rich fraction. At later stages of development the 44,000-\( M_r \) protein declined and an additional 30,000-\( M_r \) protein appeared in the wall fraction. The protein pattern of the particulate fraction was similar, although not identical, to that of the wall, raising the possibility that the former was made up of fragments of part of the wall generated during the disruption process. A developmentally regulated protein with an \( M_r \) of 24,000 was found exclusively in the soluble fraction.

The method for breaking macrocysts described here has the benefits of being simple, efficient, and reproducible and the added advantage of providing subfractions of the macrocyst. It proved to be suitable not only for \( D. \) \textit{mucoroides}, but also for macrocysts of \( D. \) \textit{discoideum} derived from the heterothallic strains V12 and NC4 and from the homothallic strain AC4 (8a). It has also been used to show that macrocyst proteinase activity (9) is not associated with the endocytes but is present in the soluble fraction (8a). While additional refinements to the method will be desirable to obtain cleaner fractions or larger-scale preparations, the procedure has already opened up the possibility of obtaining material suitable for analyzing the composition of macrocyst walls and isolated endocytes.

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REFERENCES


