RESEARCH NOTES

The Use of Density-Gradient Centrifugation for the Purification of Eggs of Meloidogyne spp.

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Investigators studying lipids, enzymes, and soluble proteins of nematodes have used plant tissue (2, 3, 4, 5, 9, 10, 12), fungi (1, 10), yeasts (1, 9), or bacteria (9) for propagating large numbers of nematodes for subsequent biochemical analyses. Although most investigators have eliminated viable bacteria from nematodes with surface sterilants, the possibility of macromolecular contamination from nonviable bacteria would remain. McClure et al. (8) recently suggested the use of a screening-centrifugation technique to obtain relatively noncontaminated eggs. In an attempt to obtain eggs of *Meloidogyne incognita* (Kofoid and White) Chitwood for analyses of structural protein, we found the above method unsatisfactory for eliminating sufficient bacterial and plant contamination. The size and density of nematode eggs are quite different from bacteria and most plant material. Therefore, density-gradient centrifugation (DGC) in combination with filtration might be a useful technique for obtaining "clean" eggs for biochemical analysis; this report describes such a technique.

Eggs of *M. incognita* were obtained from roots of tomato *(Lycopersicon esculentum* Mill. 'Bonny Best'), grown in 30-cm clay pots of Lloyd sandy clay-loam:vermiculite mixture (3:1) or builders sand in a greenhouse. Roots were collected 4-8 weeks after planting. Three methods of extracting eggs were compared: (i) the method of McClure et al. (8), (ii) McClure et al. without sodium hypochlorite (SH), and (iii) McClure et al. modified as follows: egg masses and small roots were placed in 500 ml of sterile distilled water instead of 1% SH. Eluted eggs and plant debris from a 16 μm (400-mesh) screen were centrifuged in water instead of sucrose. After centrifuging for 2 min at 1,000 $\times$ g in 15-ml conical glass tubes, all but 6 ml of the supernatant were removed with a pipette, and the pelleted eggs and host debris was stored at 4 C. Two ml of the suspensions prepared by the three methods were layered slowly onto the surface of discontinuous 60:40:20:10% (6 ml each) sucrose gradients and centrifuged for 20 min at 9,000 $\times$ g in a SW 25.1 rotor using a Beckman L-40 ultracentrifuge at 4 C.

Visual examination of the gradients after centrifugation showed the presence of two major bands. The location of the bands depended only on whether or not the eggs were extracted with SH. Bands from SH extractions were 5-10 and 13-18 mm below the meniscus and were not clearly separated (Fig. 1-A). Bands from non-SH extractions were located 15-20 and 27-31 mm below the meniscus and were clearly separated (Fig. 1-B). All gradients contained a large pellet of host debris. Sixty-six percent of the eggs in the SH gradients were in the upper band (fraction 2), and most of the plant debris was in the lower band (fraction 3) (Fig. 1-A). Most of the eggs (60%) in the non-SH extractions also were found in the upper band (fraction 2) but few were above it (Fig. 1-B). The lower band (fraction 4) contained nearly 1,000 eggs (21% of the total) but also considerable host debris. Since SH did not improve yields of eggs (263/g root with SH versus 273/g root without SH); and, since eggs not extracted with SH were more clearly separated from plant debris on sucrose-density gradients, we chose not to use SH.

The final purification step consisted of removing the eggs from the upper bands and washing them with 100 ml of 0.85% sterile saline on an 8 μm Millipore filter. Eggs were removed from the filter with 2.0 ml of sterile distilled water and stored at −40 C.

Bacterial contamination at each step in the purification was determined by pipetting samples onto the surface of triplicate nutrient agar (Difco) plates. After incubating the plates for 5 days at 30 C, numbers of bacterial colonies per plate were recorded. Results of bacterial assays of several different egg

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preparations showed the following numbers of bacteria per gram of roots extracted: method i, bacteria killed by SH; method ii, 150,000; method iii, 1,500,000; upper band from sucrose gradient (method iii) 830; and final preparation (method iii) 260. Numbers and location of bacteria in a typical gradient (extraction method iii) are shown in Fig. 1-B.

Our DGC-filtration method of obtaining large numbers of relatively 'pure' nematode eggs should be of value in determining the macromolecular composition of nematodes. When compared to the method of McClure et al. (8), the DGC step alone reduced viable bacteria nearly 200-fold. In most biochemical studies of nematodes, certain disinfectant procedures have been used to minimize viable contamination. However, interpretation of the results is still complicated by the difficulty in distinguishing between the contribution of macromolecules from nematodes and from associated nonviable bacteria, yeast, or plant material. For example, several investigations (1, 2, 3, 4, 5, 12) of nematode proteins have employed techniques capable of detecting as little as 1 μg of protein (7) yet made no mention of the origin of the protein in their samples. All bacteria contain large amounts of proteins and some contain significant amounts of lipid (6, 11).

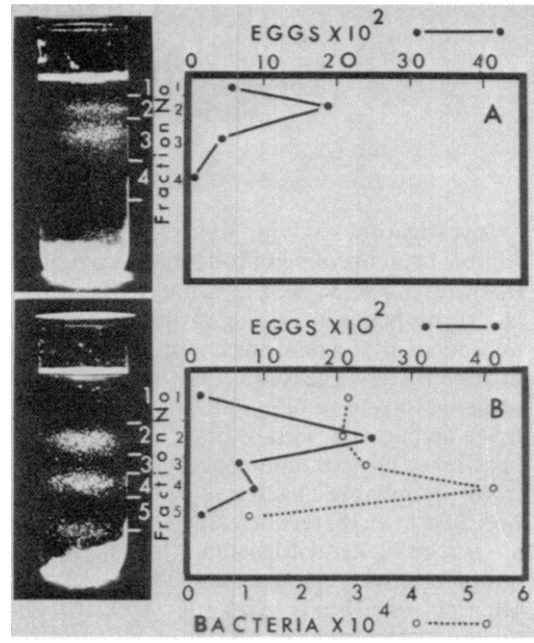

FIG. 1-(A, B). Density gradient columns and graphs showing numbers of eggs and bacteria in each fraction after centrifugation in a 60:40:20:10% sucrose discontinuous gradient for 20 min at 9,000 × *g* in a Beckman SW 25.1 rotor at 4 C. **A)** Gradients layered with 2 ml of eggs extracted with sodium hypochlorite. Bacteria killed. **B)** Gradients layered with 2 ml of eggs extracted without sodium hypochlorite.

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