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Isolation of Subcellular Granules from Second-Stage Juveniles of *Meloidogyne incognita*[1]

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Abstract: Subcellular granules from the second-stage (preparasitic) juveniles of root-knot nematode *Meloidogyne incognita* were isolated by isopycnic centrifugation on Percoll. The granules had an apparent density of 1.13 g/cm³. The relative specific activity of acid phosphatase in the granule extract was 8.4. Acid phosphatase activity was also detected histochemically in the subventral gland granules. Electron microscopy and malate dehydrogenase activity indicated that contamination of granules by mitochondria was negligible. Electrophoresis of the granule extract in the presence of sodium dodecyl sulfate showed 15–20 major protein bands.

Key words: esophageal gland, giant cell, acid phosphatase, secretory granule, root-knot nematode.

Root-knot nematodes, *Meloidogyne* species, are obligate sedentary endoparasites that parasitize more than 2,000 plant species. Second-stage juveniles penetrate root tips of susceptible plants and induce tumor-like feeding sites called giant cells (8). Giant cells differ from surrounding cells by their larger size, high rate of metabolism, dense multinucleate cytoplasm, and wall ingrowths (15).

First observed by Treub (22), giant cells have been the subject of extensive reviews (6,10,11). The nematode influences the physiology of its host by transforming normal undifferentiated root cells into the highly specialized giant cells. This change is induced by, maintained by, and is completely dependent on a continuous stimulus from the nematode. Conversely, the nematode is dependent on the giant cells for nutrition for growth, development, and reproduction. Giant cells do not divide, and removal of the stimulus leads to their atrophy (4). Secretions from three nematode esophageal glands, two subventral and one dorsal (7), are thought to be involved in the infection process and subsequent transformation of the host tissues. Bird (5) speculated that secretions from the subventral glands may be involved in juvenile hatch, juvenile penetration of roots, and initiation of the giant cells. He suggested that dorsal gland secretions have a role in the maintenance of giant cells. Secretory granules from any life stage of *Meloidogyne* species have not yet been purified so that their contents could be characterized and their role in this very specialized host–parasite relationship elucidated. We describe here the isolation of subcellular granules from the second-stage juveniles of *M. incognita.*

MATERIALS AND METHODS

Collection of juveniles: M. incognita (Kofoid & White) Chitwood was propagated

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on greenhouse-grown 'Rutgers' tomato (*Lycopersicon esculentum* Mill.). Eggs from 150–200 plants infected for 50–70 days were isolated by the method of Hussey and Barker (13). Eggs were separated from plant and soil debris by centrifugation in 20% sucrose solution and washed thoroughly on a 25-μm-pore sieve (W. S. Tyler, Inc., Mentor, Ohio) to remove the sucrose. Following surface sterilization in streptomycin sulfate (0.1%, 20 minutes) and chlorhexidine diacetate (0.2%, 20 minutes), eggs were incubated in water on 25-μm-pore sieves in plastic containers at 25 C (23). Hatched second-stage juveniles (J2) that passed through the sieve were collected daily and stored in water at 15 C. Air was bubbled through the nematode suspension daily for 10 minutes.

Isolation of subcellular granules: Pools of 1–6-day-old J2 were concentrated by low speed centrifugation (500 *g*) in water. Six to nine million J2 were resuspended in 30% sucrose and centrifuged in a swinging bucket rotor at 1,700 *g* for 5 minutes. The pellet, consisting of dead J2 and other debris, was discarded and the J2 floating on the sucrose were collected by pipet and washed twice with distilled water and twice with 15-ml aliquots of 0.01 M Tris-HCl (pH 7.2)–0.25 M sucrose (standard buffer). All subsequent operations were performed at 2–4 C. Suspended J2 were homogenized in a rotary cell homogenizer (B. Braun, Burlingame, California) with 15 ml of 1-mm glass beads at 2,000 rpm for 30 seconds. The apparatus was cooled by liquid CO_2. The homogenate was centrifuged at 750 *g* for 10 minutes. The supernatant fluid was carefully removed and the pellet rehomogenized. This process was repeated until most of the nematodes were broken as determined by examining a drop of the homogenate with a microscope. The pooled supernatant was centrifuged at 750 *g* for 10 minutes. An aliquot of this postnuclear supernatant was set aside for comparison of protein and enzyme contents. The final supernatant fluid was then made 1 mM with respect to EDTA, incubated for 10 minutes, and centrifuged at 10,000 *g* for 20 minutes. The crude granule pellet thus obtained was suspended in 7.5 ml of standard buffer, mixed with 7.5 ml of 90% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) in standard buffer, and centrifuged at 27,000 *g* for 20 minutes. A band of granules was visible at the bottom of the self-forming gradient. A parallel gradient containing only density marker beads

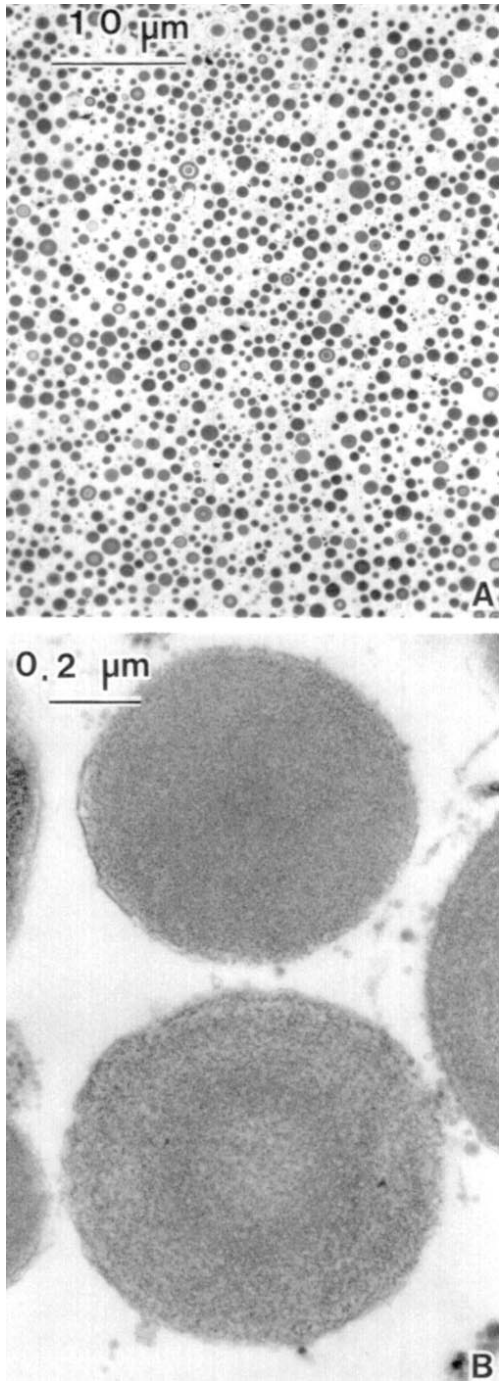
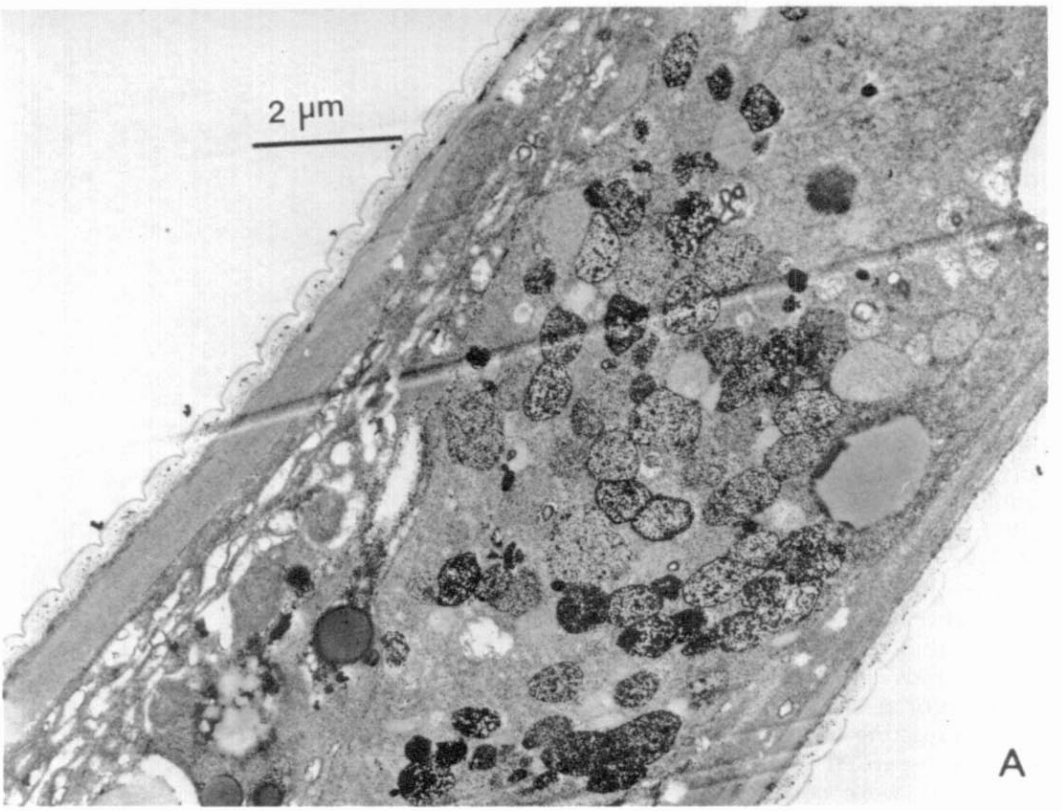
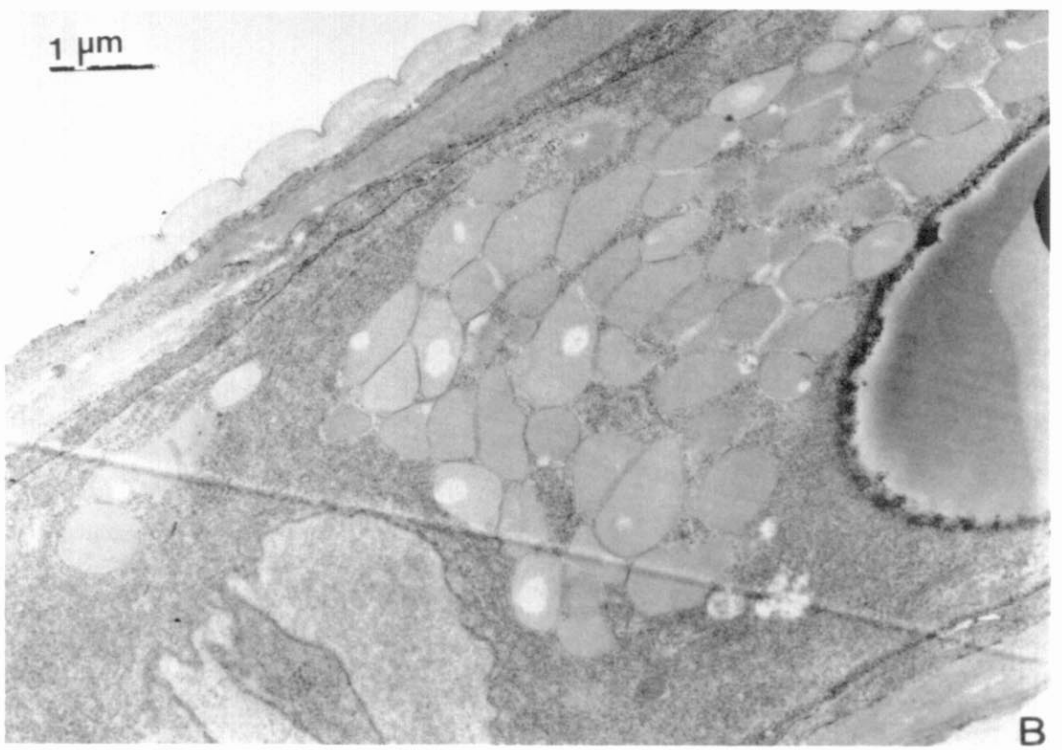


FIG. 1. Electron micrographs of the granule fraction isolated from second-stage juveniles of *Meloidogyne incognita* by density gradient centrifugation on Percoll. A) 1,700×. B) 60,000×.


2 µm
A


1 µm
B

(Pharmacia Fine Chemicals, Uppsala, Sweden) also was run routinely to determine the density of granules. Gradient material above the granule band was siphoned off, and the granules were pipetted into 15 ml of standard buffer and centrifuged at 27,000 *g* for 10 minutes. The resulting pellet was transferred into 1.5 ml of standard buffer in a 1.5-ml microfuge tube and centrifuged at 27,000 *g* for 5 minutes. The final granule pellet was either snap-frozen and stored at −80 C or fixed immediately for electron microscopy.

Electron microscopy: Second-stage juveniles of *M. incognita* were collected from egg masses and fixed in 3% glutaraldehyde in 0.1 M cacodylate (pH 7.2) at 4 C for 1 hour. After the J2 posterior sections were severed, specimens were fixed for an additional 3 hours and then thoroughly rinsed in 0.1 M cacodylate buffer containing 5% sucrose. Granule pellets isolated from J2 were fixed in 2% glutaraldehyde in 0.1 M cacodylate (pH 7.2) for 4 hours and then rinsed in 0.1 M cacodylate buffer (pH 7.2) with 5% sucrose. Juveniles and pellets were further processed by the following procedure. Postfixation was in 1% OsO_4 in cacodylate buffer for 1.5 hours at 4 C. Samples were dehydrated in an ascending series of ethanol concentrations (70–100%) and passed through two changes of propylene oxide. After infiltration in a mixture of propylene oxide and Epon (1:1), specimens were embedded in Mollenhauer's mixture of Epon and Araldite (18). Silver sections were cut on a Porter-Blum MT-2 ultramicrotome, mounted on uncoated 300 mesh grids, and stained with uranyl acetate and lead citrate. Observations and photographs were made on a Zeiss 10A transmission electron microscope operating at 60 kV.

For acid phosphatase histochemistry, freshly harvested J2 were fixed in 2% glutaraldehyde for 30 minutes and their anterior portions removed by dissection. The decapitated nematodes were further fixed in 2% glutaraldehyde for 2 hours. After rinsing in 0.05 M acetate buffer (pH 5.0) they were incubated in Gomori's (12) acid phosphatase medium for 75 minutes, rinsed in distilled H_2O, and postfixed in OsO_4. Dehydration and embedding were as described earlier. The controls lacked the substrate, β-glycerophosphate.

Preparation of granule extract: Granule pellets from several preparations were pooled and incubated in a 0.1% Triton X-100 (Bio-Rad Corp., Richmond, California) in appropriate buffers for 10–12 hours at 4 C. Following incubation, the suspension was subjected to the freeze–thaw technique used for lysing lysosomes (2). After freezing and thawing 10 times, the suspension was centrifuged at 27,000 *g* for 10 minutes. The pellet was reextracted twice with 0.1% Triton X-100 in the same buffer and centrifuged. Supernatant fluids were pooled and used for protein determination, enzyme analyses, and electrophoresis.

Assays: Protein concentrations were determined by the Coomassie Blue G-250 dye binding method of Read and Northcote (21) as described by Peterson (20) with bovine serum albumin as the standard. Acid phosphatase activity present in the granule extract was assayed by measuring inorganic phosphate (Pi) released from β-glycerophosphate at pH 4.85. The reaction mixture contained the granule extract, 0.05 M β-glycerophosphate, and 0.05 M sodium acetate (pH 4.85) in a total volume of 0.3 ml. Pi was determined by the method of Chen et al. (9) as described by Ames (1). Specific activity was defined as the number of micromoles of Pi liberated by one milligram of protein in 60 minutes at 37 C. Malate dehydrogenase was assayed according to Kitto (16), except that Tris-HCl (pH 7.5) was used instead of potassium phosphate. Final volume of the reaction mixture, 0.5 ml, contained 0.05 M Tris-HCl (pH 7.5), 0.33 mM oxaloacetate, 3.3 mM $MgCl_2$, and 0.1 mM NADH. Specific activity of the enzyme was defined as the number of micromoles of NADH oxidized per

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FIG. 2. Acid phosphatase activity in the granules of subventral glands of *Meloidogyne incognita*. A) Electron dense deposits of lead phosphate indicative of a positive reaction with substrate, β-glycerophosphate, added. B). Control nematode, without β-glycerophosphate.

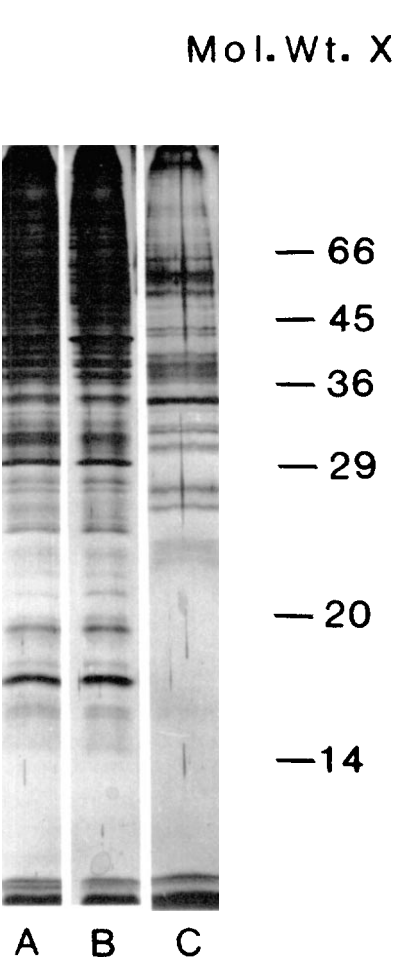

FIG. 3. SDS–polyacrylamide gel electrophoresis of granule extract from second-stage juveniles of *Meloidogyne incognita*. As described in the text, 1.1 μg total protein was electrophoresed on a homogeneous 12% gel and stained with $AgNO_3$. The gel was calibrated with molecular weight standards obtained from Sigma Chemical Company (Cat. No. MW-SDS-70L): lactalbumin (14,200); soybean trypsin inhibitor (20,100); carbonic anhydrase (29,000); glyceraldehyde-3-phosphate dehydrogenase (36,000); ovalbumin (45,000); and bovine serum albumin (66,000). A) Postnuclear supernatant prior to the sedimentation of the granules. B) Supernatant after the granules were sedimented. C) Granule fraction.

minute by one milligram of protein. Relative specific activity was defined as the ratio of enzyme specific activity in the granule extract to that in the postnuclear supernatant.

Electrophoresis: Samples containing 1.1 μg protein were electrophoresed on 12% SDS-polyacrylamide slab gels as described by Laemmli (17) and stained with $AgNO_3$ as described by Morrissey (19).

TABLE 1. Distribution of marker enzymes before and after isolation of granules from second-stage juveniles of *Meloidogyne incognita* on Percoll density gradients.

Marker enzyme	Specific activity*		Relative specific activity
	Postnuclear supernatant†	Granule fraction	
Acid phosphatase	2.50 (4)‡	21.00 (2)	8.400
Malate dehydrogenase	10.43 (4)	4.20 (4)	0.402

* Specific activity is defined as micromoles of Pi released by 1 mg of protein in 60 minutes for acid phosphatase and as micromoles of NADH oxidized by 1 mg of protein in 1 minute for malate dehydrogenase.

† Postnuclear supernatant was the homogenate after removal of cell debris and nuclei.

‡ Numbers in parentheses indicate the number of determinations.

RESULTS

Esophageal glands of *M. incognita* J2 contain numerous spherical secretory granules with an average diameter of 0.8 μm. The average size of granules isolated by Percoll density gradient centrifugation (Fig. 1) was similar to the size of the granules present in the esophageal glands of intact J2. Except for the presence of some Percoll particles that were not removed by the washings, the granule preparation appeared essentially free of other subcellular organelles. The isolated granules had an apparent density of 1.13 g/cm^3.

Electron microscopic histochemistry of intact J2 showed that granules in the subventral glands contained acid phosphatase activity (Fig. 2). Granule extracts also contained some acid phosphatase activity (Table 1). No mitochondria occurred in the isolated granule fraction (Fig. 1) and the trace levels of malate dehydrogenase (a mitochondrial enzyme) in the isolated granules (Table 1) confirmed the absence of mitochondria. The granule extract yielded 15–20 major protein bands with molecular weights ranging from 10,000 to 100,000 daltons (Fig. 3).

DISCUSSION

Percoll density gradient centrifugation has been used previously by many researchers to isolate subcellular organelles

from a variety of sources (Percoll Reference List, Pharmacia Fine Chemicals, 1983). Using this versatile medium, we have isolated subcellular granules from J2 of *M. incognita*. To our knowledge, subcellular organelles have not been isolated previously from any plant parasitic nematodes.

Enzyme analysis has indicated that granule extract contains some acid phosphatase activity. Electron microscopic histochemistry of fixed J2 demonstrated that the subventral gland granules also contain acid phosphatase activity. Ribonuclease activity was not found in granules of esophageal glands (14). These data suggest that the acid phosphatase activity may not be lysosomal in origin, since acid phosphatase and ribonuclease are two of many enzymes found commonly in lysosomes from different sources (3). The functional significance of this acid phosphatase is at present unknown.

The granule extract did not contain significant levels of malate dehydrogenase activity, indicating that the granule preparation is free of mitochondria. Malate dehydrogenase activity equivalent to that present in the original homogenate was recovered in other fractions (data not presented). The absence of mitochondria in granule preparations was confirmed by electron microscopy.

Granule extract contained approximately 0.02% of the total protein found in the crude homogenate (data not shown). Electrophoresis of granule extract revealed that the granules contain several proteins.

Secretions from dorsal and subventral esophageal glands of *M. incognita* J2 have long been considered responsible for initiating formation of the giant cells. However, a substance, protein or otherwise, of nematode origin has not been detected inside a giant cell because appropriate probes have not been available. A major goal in studies of adaptive cellular changes induced in plants by parasitic nematodes is the identification of substance(s) injected by the nematode (15). Nevertheless, little information is available on nematode secretions. Monoclonal antibodies are now being developed against the constituents of isolated granules. Immunocytochemical localization of those constituents within esophageal glands of nematodes will establish in which gland(s) the isolated granules were formed. Antibodies from positive hybridomas will also be used to detect nematode secretions in plant giant cells and to purify biologically active molecules.

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