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Production and Partial Characterization of Stylet Exudate from Adult Females of *Meloidogyne incognita*

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Abstract: Adult females of *Meloidogyne incognita* were excised from tomato roots and incubated in 0.04 M phosphate buffered saline, pH 7.4 for 18–72 hours to allow accumulation of stylet exudate. Twenty-four percent of the females produced exudate during the initial 18-hour incubation period; 70% of those females producing exudate initially produced additional exudate during the subsequent 54-hour incubation period. Analysis of exudate by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed the presence of at least nine major protein bands. Differential staining with silver and Coomassie Brilliant Blue G-250 stains indicated that three of the bands were glycoproteins. Upon acid hydrolysis, 14 amino acids were detected in the stylet exudate. The basic amino acids lysine, histidine, and arginine comprised 21.8% of the total amino acids detected. No peroxidase activity was detected in the stylet exudates. Data presented extend and generally confirm prior work on the chemical composition of stylet exudate.

Key words: root-knot nematode, biochemistry, peroxidase, amino acid, protein, stylet exudate, *Meloidogyne incognita*, giant cell.

The intimate relationship between root-knot nematodes (*Meloidogyne* spp.) and host giant cells has long been recognized (2). The mechanisms involved in giant cell formation, however, remain unclear. The consensus is that factors responsible for giant cell formation are injected into the host cell through the nematode stylet (6,19).

Linford (10) was the first to suggest a relationship between nematode feeding, stylet exudates, and giant cells. He observed and photographed an exudate that accumulated at the oral aperture of a nematode excised from host tissue and incubated in vitro. Since then, others (1,7,12) have repeated Linford's work with similar results. Such successes, however, have been attained only with adult female nematodes. No one has yet observed stylet exudate accumulation with juvenile (J2) nematodes incubated in vitro.

Alteration of normal host cells into giant cells occurs before maturation of the J2 nematodes into adult females. Histopathological studies (9,14) have shown giant cells associated with J2 nematodes. Thus, even though stylet exudates have not been observed in J2 nematodes incubated in vitro, such exudates probably do occur when the J2 nematodes are parasitic. Whereas J2 nematodes initate giant cells, adult female nematodes may only maintain the biological activity of giant cells by subsequent stimulation of affected host cells; a giant cell soon senesces when the associated nematode has been removed (2). The ability of adult females to induce giant cells has not been tested.

Because J2 nematodes maintained in vitro do not accumulate the collectable amounts of stylet exudate that adult female nematodes produce and because female nematodes very likely play a role in giant cell maintenance and activity, we initiated a program to characterize adult female stylet exudates in an effort to elucidate specific aspects of this role. A preliminary report has been presented (12).

MATERIALS AND METHODS

Cultural procedures: Populations of *M. incognita* race 3 and 4 were originally isolated from cotton, whereas the population of *M. arenaria* race 1 was isolated from peanuts. All populations were maintained on 'Rutgers' tomato (*Lycopersicon esculentum* L.); the

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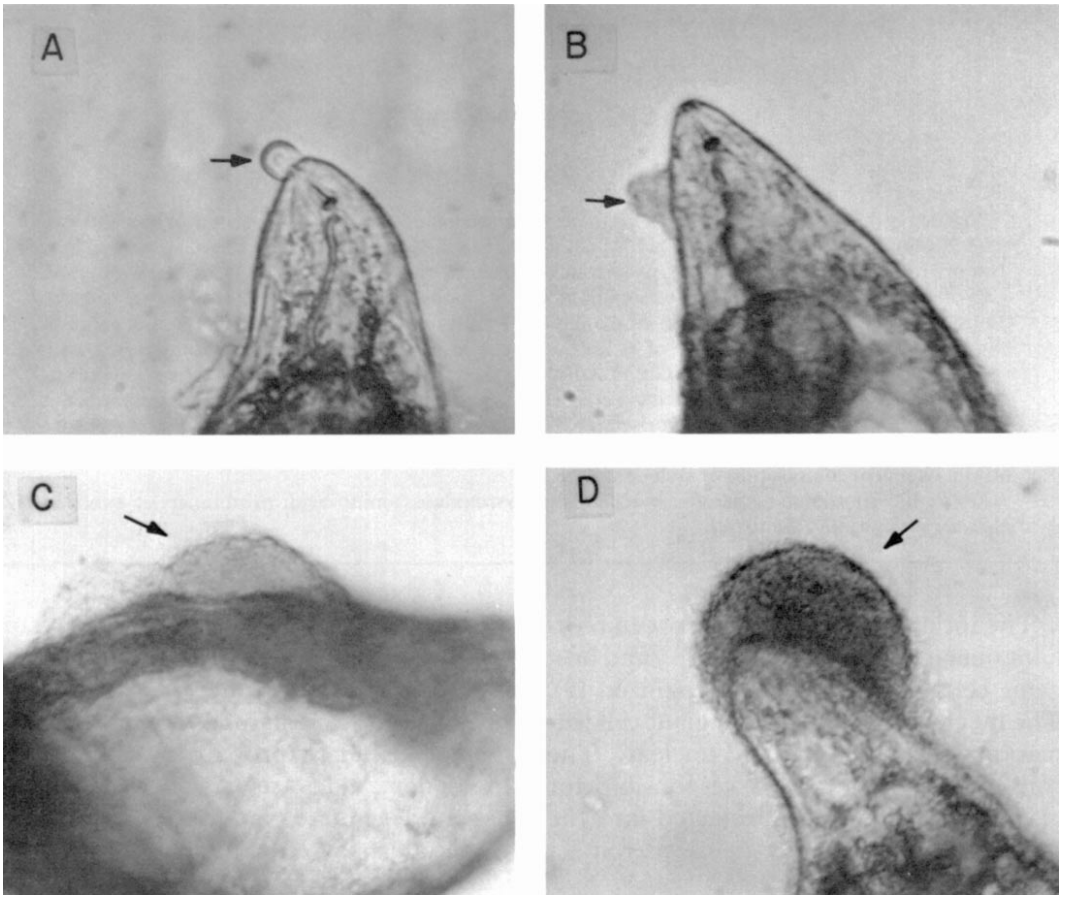

FIG. 1. Exudates associated with adult females of *Meloidogyne incognita*. A) Stylet exudate. B, C) Excretory pore secretions. D) Amalgamation of stylet and amphidial exudates.

race status of each population was reconfirmed periodically by a limited host differential test (17) that included 'NC95' tobacco (*Nicotianna tabacum* L.), 'Deltapine 16' cotton (*Gossypium hirsutum* L.), and 'Florunner' peanut (*Arachis hypogaea* L.). To obtain adult females, 4–6-week-old tomato seedlings were inoculated with eggs or freshly hatched J2 and maintained at 28 C with 14-hour days. After 4–7 weeks, infected roots were harvested and washed free of soil. Galled root pieces were placed in 0.04 M phosphate buffered (pH 7.4) saline (PBS) in petri dishes. The galled roots were examined under a dissecting scope and young adult female nematodes were carefully excised from the root tissue. These nematodes were transferred into sterile PBS containing 0.01% chlorhexidine diacetate and held for 1–3 hours at room temperature. The excised females were then transferred to wells of sterile depression slides (2–3 females per well) containing sterile PBS. The slides were placed in moisture chambers and exposed to 20-second pulses of 302-nm radiation (1.600 μWatts/cm^2/second) every hour during an 18–24-hour incubation period to suppress bacterial populations. During the incubation period, viscous-appearing materials accumulated near the head of some nematodes. Three types of accumulations occurred: stylet exudates (Fig. 1A), excretory pore secretions (Fig. 1B–C), and what appeared to be an amalgamation of stylet and amphidial exudates (Fig. 1D). Care was taken to avoid collecting other accumulations when collecting stylet exudates, which were identified based on position at the oral aperture and similarity to

previous reports of exudate appearance (27). Only those stylet exudates produced in the initial 18-hour incubation period and appearing to be free of bacterial contamination were collected for analysis.

Collecting stylet exudate: All glassware used to collect and transfer accumulated stylet exudates was siliconized with 10% Surfasil (Pierre Chemical Co., Rockford, IL). Stylet exudate accumulated during in vitro incubation was collected with glass microneedles, hand drawn from 50-μl capillary pipettes. Nematode specimens on depression slides were observed with a macroscope (Wild M 420), and the collecting needle was positioned with a micromanipulator. The accumulated exudate, and as little as possible of the surrounding PBS, was aspirated from the nematode and transferred into a sterile 25-μl microampule. Approximately 50 nl of exudate (i.e., all of the exudate collected each day) in approximately 10 μl of ambient PBS was added to each ampule. The ampules were flame sealed and stored at −70 C until used.

Efficiency of procedures: Two technicians who were dexterous at excising female nematodes from root tissue determined the ratio of excised females that accumulated stylet exudate during the first 18-hour in vitro incubation period to the total number of females excised for 10 separate groups of females. Nematodes that accumulated stylet exudate during the first 18 hours were then transferred to fresh PBS in clean depression slides, and their exudate production during a subsequent 54-hour period was determined.

Protein electrophoresis: Proteins in collected stylet exudate were separated by thin (0.5 mm) sodium dodecyl sulphate (SDS) (0.1%) polyacrylamide gel (12%) electrophoresis using a vertical BioRad system at an initial current of 30 mA. A bromphenol blue dye marked the migration of the front; running time was approximately 50 minutes at 4 C. The entire contents of three microampules (150 nl exudate, 30 μl PBS) were pooled to make one sample for electrophoretic analysis. Commercial (BioRad) protein molecular weight standards were co-electrophoresized for comparative purposes.

After electrophoresis, the gels were soaked in 50% methanol containing 0.04% formaldehyde for 1–2 hours to fix the proteins which were then stained with silver (11) or Coomassie Brilliant Blue G-250. To identify glycoproteins, selected gels were stained sequentially with silver and coomassie stains (5). Developed gels were photographed, and protein weights were estimated based on adjacent standards.

Peroxidase analysis: Several electrophoretically separated exudate samples (on 12% polyacrylamide gels without SDS) were analyzed for peroxidase activity using *o*-dianisidine or benzidine as the H^+ donor (16,18). Aqueous extracts from tomato and tobacco leaf tissues were used as positive controls for the peroxidase assay.

Amino acid analysis: Amino acids were analyzed by the method of Blackburn (3,4). Approximately 500 nl of stylet exudate in 100 μl ambient PBS was hydrolyzed in constant boiling HCl for 24 hours at 105 C. The hydrolysate was evaporated to dryness with argon and dissolved in 60 μl 0.2 M lithium citrate injection buffer, pH 2.2. Amino acid identification and quantitation were obtained with a Beckman Model 4400 amino acid analyzer interfaced to a Hewlett Packard 3300A computer. Amino acid resolution (physiological mode) using 1.6 M lithium citrate buffer (pH 3.55) was 0.5–30.0 nM.

Results

Efficiency of procedures: The efficiency of excision of adult female nematodes from host roots was related to age of the infected roots. It was more difficult to excise nematodes from roots infected more than 6 weeks than from roots infected 4–6 weeks. About 50 adult females could be excised per hour from 4–6-week-old infections. A higher rate of excision could be obtained, but the percentage of females producing exudate was reduced.

During the first 18-hour in vitro incubation period, 24% of the excised nematodes accumulated observable (at 30×

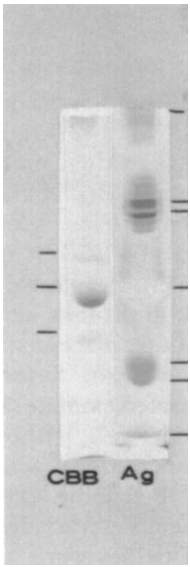

FIG. 2. Protein bands in exudate of *Meloidogyne incognita* females detectable with silver (Ag) or Coomassie Brilliant Blue G-250 (CBB) stains following electrophoretic separation on sodium dodecyl sulfate-polyacrylamide gels. Bars indicate location of major bands.

magnification) stylet exudate. Of these nematodes, 79% accumulated stylet exudate during the next 24-hour incubation period; of the latter, 74% accumulated exudate during the third 24-hour incubation period. Nematodes that failed to accumulate exudate during the initial incubation period generally did not accumulate exudate during subsequent incubations. After 3 days of in vitro incubation, the apparent (under 30× magnification) condition of the nematodes was poor, and they were heavily contaminated with bacteria.

The average volume of stylet exudate per nematode, assuming that exudates had the dimensions of a sphere, was 0.49 nl. Therefore, assuming average conditions for all aspects of the exudate collecting process (50 worms excised per hour of which 24% accumulate exudate), one person can collect about 50 nl of exudate in 8 hours.

Protein electrophoresis: Nine protein bands were routinely (10 replications) detected by silver and Coomassie Brilliant Blue stains after electrophoresis of *M. incognita* race 3 stylet exudates (Fig. 2). Three protein bands were not detected with silver stain, but were visualized with Coomassie Brilliant Blue. No differences in protein patterns were detected among races 3 and 4 of *M. incognita* and race 1 of *M. arenaria.* Based upon the molecular weights of commercial standards, the proteins in stylet exudate ranged from 25 k to 125 kdaltons.

Peroxidase analysis: Peroxidase activity was not detected in stylet exudates after electrophoresis on polyacrylamide gels, or in exudate in situ in nematodes incubated in depression well slides. A positive reaction for peroxidase activity was obtained after electrophoresis of tomato or tobacco leaf extracts.

Amino acids: Fourteen common amino acids were detected in the acid hydrolysate of the stylet exudates (Table 1). The basic amino acids lysine, histidine, and arginine comprised 21.8% of the total amino acids detected.

DISCUSSION

A major obstacle to the elucidation of the complex series of events that result in giant cell formation and maintenance in host roots in response to infection by *Meloidogyne* has been the inability to isolate and characterize components from the nematode that induce these events. Working with adult female nematodes, we have

TABLE 1. Amino acid composition of stylet exudate from *Meloidogyne incognita* adult females.

Amino acid	p mole/female†
Lysine	154
Serine	111
Alanine	101
Leucine	99
Proline	92
Aspartic acid	91
Glutamic acid	89
Glycine	83
Arginine	62
Valine	60
Phenylalanine	46
Histidine	44
Tyrosine	37
Isoleucine	19

† Based on ca. 500 nl of exudate from 100 nematodes.

developed procedures that allow collection of sufficient amounts of stylet exudates for at least partial characterization.

Our data indicate that the stylet exudates of *Meloidogyne* species contain a complex of several proteins. Based on differential staining, three of the proteins may be glycoproteins (5). These data are consistent with earlier observations by Bird (1) that the stylet exudates had characteristics of proteins (based on microspectrophotometric analysis) and carbohydrates (based on histochemical tests). Bird also suggested the presence of histone-like proteins in the stylet exudates; our finding that the basic amino acids lysine, histidine, and arginine comprise 21.8% of the total amino acids detectible in the stylet exudate is consistent with Bird's observations.

Hussey (7) reported the presence of peroxidase activity in homogenates of adult females of *M. incognita* and presented histochemical evidence for peroxidase activity in stylet exudates from adult females. Others (8,16) have suggested that the peroxidase activity in homogenates is predominately of host origin and was ingested during nematode feeding. In the present study, we were unable to detect any peroxidase activity in stylet exudates of *M. incognita* following electrophoresis.

It would be premature to speculate on the precise role of the stylet exudates in giant cell development and activity (2). Our working hypothesis is that only the J2 stages of the nematodes are able to initiate giant cell formation, whereas adult female nematodes are required to maintain the physiological activity of previously induced giant cells. If only the J2 stages of the nematodes are capable of initiating giant cell formation, then it is likely that the host specificity characteristic of *Meloidogyne* populations is determined by the events occurring with this stage and not at the stage of giant cell maintenance by the adult female nematodes. Our inability to detect differences among the stylet exudates of two races of *M. incognita* and one race of *M. arenaria* is consistent with this hypothesis. It also is possible that the stylet exudates from adult females are involved in the formation of feeding tubes (15) that develop within the giant cells.

Regardless of the role of stylet exudate in the host–parasite relations of *Meloidogyne* spp., additional work is needed to further characterize the chemical composition of the stylet exudate and determine its precise origin. To date there is no conclusive evidence that "stylet exudates" originate solely from the stylet. Work is in progress to determine if these exudates arise from secretory granules produced within the nematode's esophageal glands (2,6) and (or) whether amphidial secretions may contribute to the stylet exudate. Reddigari et al. (13) have reported the isolation of subcellular granules from preparasitic *M. incognita* J2; these granules are ultrastructurally similar to secretory granules from the esophageal glands. It will be important to determine if stylet exudate is derived only from secretory granules and the extent of similarity that exists between gland products produced by J2 and adult stages of the nematode.

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