

Disc-Electrophoretic Studies of Soluble Proteins and Enzymes of *Meloidogyne incognita* and *M. arenaria*¹

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Abstract: Soluble-protein and eight enzyme profiles obtained by polyacrylamide-gel electrophoresis were compared between *Meloidogyne incognita* and *M. arenaria*. Esterase, malate dehydrogenase, and α -glycerophosphate dehydrogenase patterns were distinctly characteristic for each species. Peroxidase and α -glycerophosphate dehydrogenase isoenzyme patterns varied when nematodes were propagated on different host plants. Similar profiles were obtained for two populations within each species. Antigenic proteins of these two species were compared following separation by electrophoresis. **Key words:** peroxidase, root-knot nematodes, characterization, antigens.

Electrophoresis of proteins is a promising tool for identification and characterization of nematodes (1, 4, 5, 6, 7, 10, 18). Soluble

protein and certain enzyme patterns were characteristic for the genera *Meloidogyne*, *Heterodera*, *Ditylenchus*, and *Aphelenchus* (4, 5). Interspecific differences in the genus *Meloidogyne* were also noted for certain electrophoretic patterns (4, 5). Ishibashi (12) reported that certain enzyme patterns of *M. incognita* varied, depending on the host plant used for propagating the nematodes. He also reported multiple forms of lactate dehydrogenase for *M. incognita*. These results were different from results obtained by Dickson *et al.* (4).

Variation in results of electrophoretic analyses of nematode proteins can occur for many different reasons; e.g., (i) methods of collecting and storing nematodes; (ii) stage of

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nematode development; (iii) protein extraction procedure; (iv) storage of protein extract; and (v) methods of enzyme analysis. All of these may have a profound influence on either the electrophoretic mobility of the proteins, the number of isoenzymes that can be detected, or both. Results of protein analyses by electrophoresis obtained by different investigators will be useful for nematode taxonomic and phylogenetic studies only if standardization of procedures is adopted; otherwise meaningful comparisons cannot be made. Chrambach and Rodbard (2) recently discussed the versatility of polyacrylamide-gel electrophoresis and the conditions necessary for pattern reproducibility.

The objectives of our investigation were: to improve methods for electrophoretic analyses of proteins of *Meloidogyne* species; to determine if differences can be detected between nematode populations; to determine if the host plant influences nematode enzyme patterns; and to compare antigenic proteins of two *Meloidogyne* species.

MATERIALS AND METHODS

Preparation of protein extract. Adult females of two populations each of *Meloidogyne incognita* (from Taiwan and Peru) and *M. arenaria* (from Virginia and Greece) were obtained as described previously (8). Adult female nematodes were washed with distilled water and suspended (1:1.5 v/v) in cold buffer at pH 7.4, freshly prepared from 0.05 M potassium phosphate, 0.85% NaCl, and 0.001 M MgCl₂. Nematodes were homogenized with the aid of powdered glass (200 mesh) in a Con-Torque® (Eberbach Corp., Ann Arbor, Mich.) powered Ten-Broeck tissue homogenizer cooled in an ice-water bath. The homogenate was centrifuged at 20,000 *g* for 30 min at 4 C, and the supernatant fluid was used as the source of proteins. During centrifugation, a viscous lipid layer collected on top of the supernatant fluid. Therefore, the supernatant fluid was carefully removed with a Pasteur pipette to prevent mixing with the lipid layer. Amount of proteins in the preparations was determined by the method of Lowry *et al.* (14) on trichloroacetic acid precipitate dissolved in 0.1 N NaOH with bovine albumin as the standard. Protein preparations were stored on ice and used within 48 hr after homogenization.

Electrophoresis. Proteins of the two *Meloidogyne* species were separated by

polyacrylamide-gel electrophoresis (3, 15). Sucrose (60 mg/ml) was added to the protein preparations to increase the density of the solution which then was layered on top of the spacer gel. Purified acrylamide (Ortec Inc., Oak Ridge, Tenn.) was used in these experiments. Since the concentration of the proteins in the preparations varied from 3.9-5.5 mg/ml, the volume of the preparation layered on top of the spacer gel was adjusted (35-50 μ liters) in order to apply approximately 200 μ g of proteins/glass column of 5 mm i.d. Electrophoresis was conducted in a Polyanalyst® (Buchler Instruments Division, Fort Lee, N.J.) at 3 ma/column in an anionic system at 4 C and terminated when the marker dye migrated 50 mm in a 7% separating gel.

Soluble protein and enzyme analyses. Soluble proteins referred to in this paper are proteins soluble in dilute aqueous salt solutions (buffer). Soluble-protein patterns were developed by electrophoretically destaining gels which had been incubated for 16-24 hr in a solution of 0.5% Buffalo Black NBR (a non-specific protein stain) in 7% acetic acid. Destained gels were scanned at 595 nm with a Gilford spectrophotometer Model 240 (Gilford Instruments Laboratories, Inc., Oberlin, Ohio).

Sites of enzymes were determined by incubating gels after electrophoresis in freshly prepared substrate solutions.

Demonstration of malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) activities were made using the method of Laycock *et al.* (13). This method, with the substitution of DL- α -glycerophosphate disodium (5 mg/ml) for L-malic acid was used to detect α -glycerophosphate dehydrogenase (α -GPDH) activity. For the detection of glucose-6-phosphate dehydrogenase (G6PDH) or 6-phosphogluconate (6-PDH), nicotinamide adenine dinucleotide phosphate and D-glucose-6-phosphate disodium (5 mg/ml) or 6-phosphogluconate (5 mg/ml) in 0.05 M Tris-HCl (pH 7.0) were appropriately substituted in the procedure.

Peroxidase activity was detected with a benzidine-guaiacol cosubstrate mixture (16). Other cosubstrates and incubation procedures previously described were also used to confirm the peroxidase activity (10, 11). Catalase activity was determined with the starch-potassium iodide procedure (11). Non-specific esterase activity was detected by incubating gels in the dark at 25 C in a solution

of 50 ml of 0.04 M Tris-HCl (pH 6.6), 1 ml of 1% α -naphthylpropionate, and 25 mg of Fast Blue RR. Better results were obtained in preliminary tests with α -naphthylpropionate as the substrate than with α -naphthyl acetate, α -naphthylbutyrate, or naphthyl AS-D acetate. Prior to incubation in a substrate medium for determining acid phosphatase (AcP) activity (17), gels were first incubated in 0.2 M acetate buffer (pH 5.0) for 15 min.

Controls consisted of gels heated in test tubes in boiling water for 10 min or gels incubated in a reaction mixture minus the substrate. Electrophoretic mobility (E_f) values were determined directly on the gel columns by comparing the migrating distance of the protein band with that of the bromophenol blue marker dye.

Influence of host plant. Enzyme analyses were made on protein preparations from the Taiwan population of *M. incognita* propagated on two additional hosts, *Nicotiana tabacum* L. 'Hicks', and *Phaseolus vulgaris* L. 'Burpee's Stringless'.

Antigenic proteins. Nematode antisera and agar medium used in these tests were prepared as described by Hussey (9). Polyacrylamide-gel columns after electrophoresis were placed in 100-mm plastic petri plates containing 15 ml of molten agar medium. Antisera was added to troughs cut parallel to and 1 cm from the embedded gel column 1.5 hr after the medium solidified. Plates were incubated at 24.5-25.5 C in a moist chamber and examined after 48 hr.

RESULTS

Soluble-protein and enzyme analyses. Characteristic soluble-protein patterns were obtained for *M. incognita* and *M. arenaria* (Fig. 1). Many of the soluble proteins from both species, however, had similar migration rates as determined when samples were separated in the same electrophoretic run. Although 30 protein bands were visible in the stained gels, only 25 and 21 protein bands were recorded by the spectrophotometer for *M. incognita* and *M. arenaria*, respectively. The last peak on the right of each scan represents a protein that migrated with the marker dye. The major protein bands recorded in the scans were reproducible. Some variation in migration rates, however, occurred among electrophoretic runs with some of the weakly stained proteins. Due to some variation in the number of minor bands, the total number of stained proteins visible in the gels ranged from 24 to 32 for *M. incognita*. Similar variation in number of visible protein bands was observed after separation of proteins of *M. arenaria*. Soluble-protein profiles for the two populations within each species were similar.

Characteristic esterase, MDH, and α -GPDH patterns were demonstrated for these two species. Although the same number of sites of esterase activity were detected for both species, characteristic differences did occur with respect to the relative activity at certain sites and E_f values (Fig. 2). The fastest migrating esterase for *M. incognita* had an average E_f of 0.41, whereas an E_f of 0.46 was obtained for the

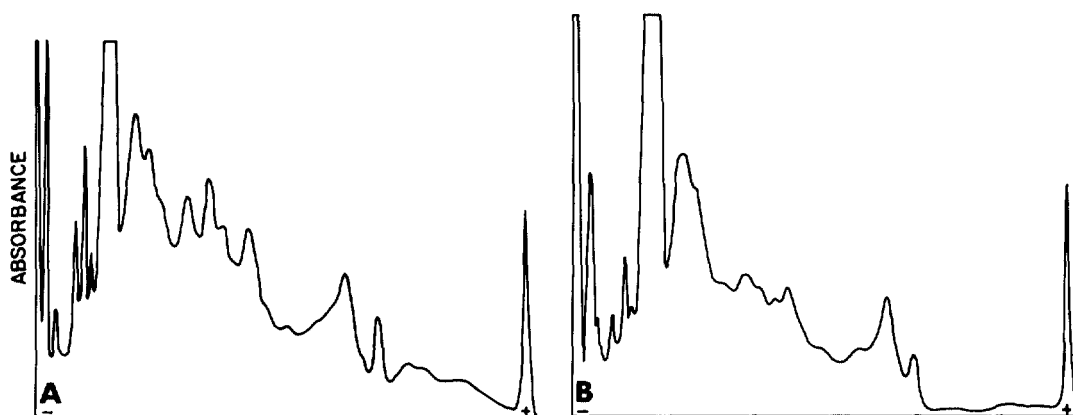


FIG. 1. Scans of profiles of soluble proteins from adult females of A. *Meloidogyne incognita* populations from Peru and Taiwan; and B. *M. arenaria* populations from Greece and Virginia, separated by polyacrylamide-gel electrophoresis. Last peak on the right of each scan represents a protein band that migrated with the tracking dye.

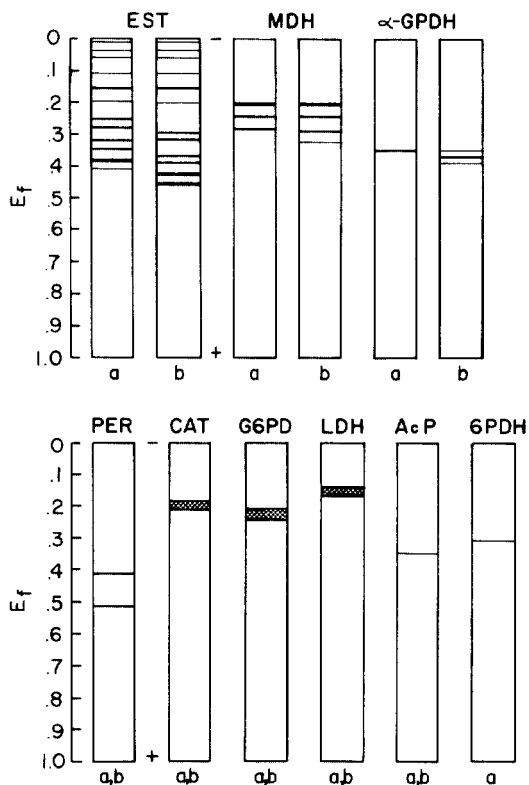


FIG. 2. Enzyme patterns obtained by electrophoretic separation of proteins of adult females of (a) *Meloidogyne incognita* populations from Peru and Taiwan; and (b) *M. arenaria* populations from Greece and Virginia. EST = esterase; MDH = malate dehydrogenase; α GPDH = α -glycerophosphate dehydrogenase; PER = peroxidase; CAT = catalase; G6PD = glucose-6-phosphate dehydrogenase; LDH = lactate dehydrogenase; AcP = acid phosphatase; and 6PDH = 6-phosphogluconate dehydrogenase. The amount of activity detected at each site is represented by the differences in the width of the individual bands, with the exception of CAT, LDH, and G6PD, where the dotted bands indicate these activities were diffuse in the gels.

fastest migrating esterase of *M. arenaria*. One site (E_f 0.39) with strong esterase activity was detected for *M. incognita*, whereas two sites (E_f 0.43 and 0.46) with strong activity were detected for *M. arenaria*. Six and five sites of weak esterase activity were observed for *M. incognita* and *M. arenaria*, respectively (Fig. 2). In two separate experiments after separation of proteins of *M. arenaria*, two esterase sites (E_f 0.31 and 0.38) normally having medium activity had weak activity. In several experiments with both populations of each species, some of the sites with weak esterase

activity were not observed. No staining was observed in control gels.

After electrophoretic separation of extracts of *M. arenaria*, four and three sites of activity were detected for MDH and α -GPDH, respectively (Fig. 2). Separation of extracts of *M. incognita*, however, revealed only three sites of MDH activity and one site of α -GPDH activity.

Profiles obtained for the other enzymes, with the exception of 6-PDH which was only tested for *M. incognita*, were similar for both species and populations (Fig. 2). All enzyme profiles for the two populations within each species were similar.

The activity of the peroxidase isoenzymes varied slightly when different phenols and aromatic amines were used as cosubstrates. In general, the isoenzymes had greater activity with caffeic acid, chlorogenic acid, and *p*-phenylenediamine than with catechol, hydroquinone, and gallic acid. In certain experiments, weak activity also occurred immediately above each of the two peroxidase isoenzymes. This type of activity was inconsistent and, therefore, was not considered to be the result of additional peroxidase isoenzymes. Strong peroxidatic activity was detected for the catalase with gallic acid, pyrogallol and hydroquinone.

The activities for three enzymes, CAT, G6PDH, and LDH, were diffuse in the gels, indicating that these enzymes were not migrating in a compact band. The activities of two of these enzymes, CAT and G6PD, were strong. Two enzymes, LDH and 6PDH, were detected only in approximately 50% of the experiments. One site of AcP activity was detected; this was obtained when gels were first incubated in 0.2 or 0.05 M acetate buffer (pH 5.0) for 15 min. When first incubated in 0.2 M buffer for 3 hr at 4 C, however, a different and more diffuse site of activity (E_f 0.16-0.20) was observed.

The profile for each enzyme was reproducible for both *Meloidogyne* species; however, variation in migration rates existed between different experiments even though procedures were standardized. This variation occurred for all the isoenzymes within a single pattern. For example, the variation among E_f values for α -GPDH of *M. incognita* in four experiments was $\pm 0.03 E_f$ units. Therefore, E_f values used in the text, with the exception of those presented for enzymes from nematodes

propagated on the different host plants, are an average of values obtained from electrophoretic analyses of a minimum of three different extracts.

Influence of host plant. The enzyme profiles obtained for nematodes propagated on tobacco were similar to those from nematodes propagated on tomato with the exception of peroxidase. Peroxidase activity was not detected in gels after separation of proteins of nematodes propagated on tobacco. When the same nematode was propagated on bean, differences were noted with two enzyme patterns. One site of weak peroxidase activity was detected at E_f 0.42, and an additional site of weak α -GPDH activity was detected at E_f 0.32.

Antigenic proteins. Thirteen immunoprecipitates developed with *M. incognita* antiserum and 12 formed with *M. arenaria* antiserum in an immunodisc electrophoresis test (Fig. 3). Based on band position, most of the immunoprecipitates were of common origin. The most intense immunoprecipitate was formed at the anodic end of the gel adjacent to the marker dye, and the largest number of immunoprecipitates were formed adjacent to the upper half of the gel. No immunoprecipitates were formed when normal serum was used.

DISCUSSION

The results of this study confirm earlier reports (4, 5, 6) that polyacrylamide-gel electrophoresis of proteins can be used to identify specific enzymes and aid in the taxonomy of nematodes. *Meloidogyne incognita* and *M. arenaria* can be distinguished reliably by their esterase, MDH, and α -GPDH patterns and less reliably by their soluble-protein patterns. Although differences were present in the soluble-protein patterns between these two species, these differences were not as striking as the differences in enzyme patterns.

Electrophoresis was superior to serological analyses of proteins for detecting differences between *M. incognita* and *M. arenaria*. In serological tests, one immunoprecipitate was unique with *M. incognita*, and eight immunoprecipitates, based on band position and coalescence, were common for both species when antigens of these two species were tested against *M. incognita* antisera (9).

Very similar soluble-protein and enzyme

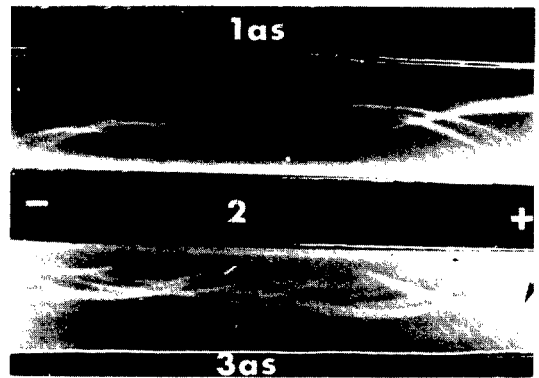


FIG. 3. Serological detection of proteins, separated by polyacrylamide-gel electrophoresis, from adult females of *Meloidogyne incognita* using homologous antiserum and antiserum to *M. arenaria*. A gel column (2) containing separated proteins of *M. incognita* is embedded in an agar medium between a trough containing homologous antiserum (1as) and one containing *M. arenaria* antiserum (3as). Arrow points to an immunoprecipitate which formed in the agar medium adjacent to the anodic end of the gel.

profiles were obtained for the two populations of each *Meloidogyne* species even though the populations originated from widely separated geographical regions of the world and had slightly different host ranges. Evans (6), however, reported differences in esterase, amylase, and acid phosphatase profiles among seven isolates of *Aphelenchus avenae*.

Comparison of the results of this study with those reported by Dickson *et al.* (4, 5) for *M. incognita* and *M. arenaria* indicates the need for careful standardization of procedures. Differences in certain E_f values and number of isoenzymes detected were found between the two studies. Since some uncontrollable variation in E_f values was observed in this study, the number of forms of an enzyme detected was considered the most reliable characteristic for distinguishing these two species. More sites of esterase activity for *M. incognita* and *M. arenaria*, and two additional sites of α -GPDH activity for *M. arenaria*, are reported in this study than were previously reported by Dickson *et al.* (4). The populations they investigated were different from those we used. Although scans of soluble-protein profiles for the same species were similar to those of Dickson *et al.* (5), a larger number of proteins are represented in our scans (Fig. 1). Apparently, more proteins were extracted with the method reported here.

Ishibashi (12) reported the detection of

multiple forms of LDH for *M. incognita*; the number and migration rates of the LDH isoenzymes differed markedly from those reported herein and by Dickson *et al.* (4). In our study, only one site of LDH activity was detected, whereas Ishibashi (12) reported 5-6 sites of LDH. He also reported esterase activity near the anodic end of the gel; in our study, esterase activity was only detected in the upper half of the gel. In the same study, some variation occurred in esterase profiles when nematodes were propagated on different host plants. This was contrary to that reported by Dickson *et al.* (4). In our study, no differences were found in esterase patterns of *M. incognita* propagated on three different host plants. Differences were detected, however, in peroxidase and α -GPDH activities. The changes in peroxidase activity are particularly interesting. These changes suggest that nematode peroxidase may have a role in the establishment of the host-parasite relationship, perhaps in initiating giant cell formation in certain host plants. Furthermore, if peroxidase from *Meloidogyne* is determined to be involved in host-parasite relationships, this enzyme could also be one of the important factors responsible for eliciting a resistant (necrotic) reaction in certain plants. Actual secretion of peroxidase by a nematode would have to be demonstrated before this enzyme could be implicated. Hussey and Krusberg (10) did not detect any peroxidase activity in extracts of *Ditylenchus dipsaci* after separation by polyacrylamide-gel electrophoresis. Two catalase isoenzymes, however, were detected and both possessed strong peroxidatic activity. Dickson *et al.* (4) did not find any differences in α -GPDH profiles when nematodes were propagated on tobacco, cucumber, or wheat. In the present study, differences were detected when nematodes were propagated on bean, but not when propagated on tobacco. Raising *A. avenae* under different cultural conditions had little influence on soluble-protein and enzyme profiles (6). Therefore, host influence on either nematode enzyme production, activity, or both varies with enzymes and host plants.

The exact nature of the phosphatase activity, either acid or alkaline, is still uncertain. Dickson *et al.* (4) detected acid and alkaline phosphatase activity at the same site in the gels. In our study, only acid phosphatase activity was detected; no activity was detected with an alkaline phosphatase reaction mixture

(17). Slightly different reaction mixtures were used in our study than were used by Dickson *et al.* (4) in an attempt to elucidate the type of phosphatase activity. Activity similar to that reported by Dickson *et al.* (4), however, was obtained when their reaction mixtures were used.

The reaction of proteins of *M. incognita* with homologous and *M. arenaria* antisera demonstrates that most proteins of *M. incognita* and *M. arenaria* are antigenically similar. A greater number of immunoprecipitates were formed with the immunodisc electrophoresis procedure than with gel diffusion tests (9). The former technique may be more valuable for demonstrating serological differences between nematode species or populations.

In the present study, only soluble anionic proteins were investigated; other differences might be detected if cationic proteins were to be examined. The capability of polyacrylamide-gel electrophoresis to differentiate and characterize nematode species makes it a useful technique; through its application, taxonomic ranks can be better defined. The possibility, however, of detecting differences in enzyme profiles of nematode populations that are associated with differences in pathogenicity, although potentially very valuable, still needs to be demonstrated.

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