Surface Coat of *Meloidogyne incognita*[1]

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Abstract: The nematode surface coat is defined as an extracuticular component on the outermost layer of the nematode body wall, visualized only by electron microscopy. Surface coat proteins of *Meloidogyne incognita* race 3 infective juveniles were characterized by electrophoresis and Western blotting of extracts from radioiodine and biotin-labeled nematodes. Extraction of labeled nematodes with cetyltrimethylammonium bromide yielded a principal protein band larger than 250 kDa and, with water soluble biotin, several faint bands ranging from 31 kDa to 179 kDa. The pattern of labeling was similar for both labeling methods. Western blots of unlabeled proteins were probed with a panel of biotin-lectin conjugates, but only Concanavalin A bound to the principal band. Nematodes labeled with radioiodine and biotin released ^{125}I and biotin-labeled molecules into water after 20 hours incubation, indicating that surface coat proteins may be loosely attached to the nematode. Antiserum to the partially purified principal protein bound to the surface of live nematodes and to several proteins on Western blots. Differential patterns of antibody labeling were obtained on immuno-blots of extracts from *M. incognita* race 1, 2, and 3; *Meloidogyne hapla* race 2; and *Meloidogyne arenaria* cytological race B.

Key words: cuticle, electron microscopy, immunology, lectin, *Meloidogyne arenaria, M. hapla, M. incognita, Meloidogyne* spp., protein, root-knot nematode, surface coat.

The extracuticular component on the outer surface of the plant-parasitic nematode is termed a surface coat (SC) or glycocalyx (35). Cetyltrimethylammonium bromide (CTAB) and other cationic detergents efficiently dissolve the SC (19,28,29), but anionic ones are less effective, perhaps owing to the strong negative charge of the surface (25). Surface coat proteins are heterogenous with regard to molecular weight (16). Glycosylation of the SC, which was detected by a variety of lectin conjugates, has been shown in a number of nematodes (12,21,33,34,36–38). The entire surface, or parts of it, were labeled with the lectin (21,34) and labeling was stage-specific (34). Surface carbohydrates appear to be part of glycoproteins in the animal-parasitic nematodes, free-living nematodes, or plant-parasitic nematodes, such as *Tylenchulus semipenetrans* and *Anquina* species (3,33).

The purpose of this investigation was to characterize the surface coat of *Meloidogyne incognita* race 3 by electron microscopic and electrophoretic analyses of labeled and unlabeled SC proteins. A parallel and collaborative investigation, using *M. javanica* as the principal test species, was conducted by researchers at the Volcani Center in Israel. It will be reported elsewhere.

Materials and Methods

Egg masses of *Meloidogyne* spp. were obtained from J. N. Sasser, Department of Plant Pathology, North Carolina State University, and propagated on eggplant, *Solanum melongena* cv. Black Beauty, in the greenhouse. The origin and identity of these populations have been reported (5). Eggs were collected from infected plants (18) and hatched on nylon mesh (pore size 20 μm) in distilled water to obtain infective second-stage juveniles (J2).

Electron microscopy: Approximately 1,000 J2, freshly hatched from eggs, were incubated in distilled water at 14 °C for 3 days to permit accumulation of SC materials. After incubation, they were washed three times with distilled water, concentrated by centrifugation at 1,000g, and divided into two equal lots.

One lot was incubated in cationized ferritin, 1 mg/ml for 2 hours at 37°C, rinsed three times with distilled water, and resuspended in 0.06 M phosphate buffer. The

ferritin-treated J2 were then transferred to special processing chambers (20); fixed for 90 minutes at 21-24 °C in 0.06 M phosphate buffer, pH 7.3, containing 3% glutaraldehyde; rinsed three times with PBS; postfixed for 1 hour at 21-24 °C with 1% osmium tetroxide; dehydrated in acetone (10% increments); and infiltrated with Spurr's low viscosity resin in acetone (10% increments). Infiltrated specimens, in undiluted resin, were cast between two glass microscope slides (30) and cured at 60 °C for 18 hours. Longitudinal silver sections of embedded J2 were mounted on uncoated copper grids, stained for 20 minutes with 2% uranyl acetate, poststained with Reynold's lead citrate, and photographed on a Hitachi H-500 TEM at 75 kV.

The other lot of J2 was stained with ruthenium red, purified, and prepared in 0.2 M cacodylate buffer containing 3.6% glutaraldehyde according to Luft (15). Stained J2 were post-fixed for 3 hours at 25 °C with 1.5% osmium tetroxide in 0.06M cacodylate buffer, also containing 500 ppm ruthenium red. They were then rinsed three times with distilled water, dehydrated, and processed for electron microscopy as described above.

Nematode surface labeling: For labeling with biotin, J2 were washed several times in distilled water and incubated with either 0.3 mg/ml biotin-N-hydroxy-succinimide-ester (Biotin-NHS) or Biotin-X-NHS, a water-soluble formulation (Calbiochem-Novabiochem, San Diego, CA), for 1 hour at 25 °C. Excess biotin was removed by centrifuging and resuspending the biotinylated nematodes four times in distilled water (13).

Radioiodination of the SC was accomplished by exposing J2 to ^{125}I-Bolton Hunter reagent according to the supplier's instructions (E.I. Du Pont de Nemours, Wilmington, DE). The reaction was carried out at pH 8.0 in 0.01 M phosphate buffered saline (PBS) with 250 μCi of ^{125}I for 1 hour at 25 °C. At the end of incubation, uncombined iodine reagent was removed by reaction with excess lysine and

the nematodes were washed repeatedly with PBS (31).

Release of labeled surface molecules: Approximately 0.2 ml of ^{125}I-labeled J2 were washed on a 4 μm-pore-size filter using distilled water to remove unbound label. Eluate was assayed for radioactivity by passing it through a 0.22-μm Ultrafree filter (Millipore, South San Francisco, CA) in a centrifuge at 2,000g for 1 minute and counting gamma emission from 1.0 ml in a scintillation vial containing 20 ml of water-miscible scintillation cocktail (ICN Biomedicals, Costa Mesa, CA). Washing to remove unbound ^{125}I continued until the counts stabilized at approximately 21,000 cpm. After washing, the volume of the solution containing the labeled nematodes was brought up to 2 ml by adding distilled water and the suspension was incubated in a 15-ml glass tube with slow agitation at 23 °C. Aliquots were collected at 0-, 1-, 2-, 4-, 8-, 16-, and 20-hour intervals. At each interval, the nematodes were pelleted and 1 ml of supernatant was passed through a 0.22-μm filter by centrifugation at 1,000g. The filtrate was placed in a scintillation vial containing 20 ml of scintillation cocktail and assayed for gamma emission. In order to maintain a constant volume, 1.0 ml of distilled water was added to the glass tube containing the labeled nematodes, and the nematodes resuspended by agitation.

Release of SC materials also was demonstrated by incubating 0.2 ml of biotinylated, and thoroughly washed, J2 for 18 hours in distilled water with gentle agitation. The supernatant was collected by centrifugation and concentrated by ultrafiltration to approximately 30 μl. Biotinylated proteins in the concentrate were separated by electrophoresis and visualized on Western blots as described below.

Solubilization of SC protein: About 1 ml (approximately 2.5×10^6) of pelleted J2 (biotinylated, radiolabeled, or not labeled) was collected as described above and washed by centrifugation in distilled water several times at 1,000g for 3 minutes. Four milliliters of 0.25% CTAB containing proteinase inhibitors (5 μg/ml aprotinin, 2 mg/

ml iodoacetamide, 0.2 mg/ml phenylmethylsulfonyl fluoride, and 50 μg/ml soybean trypsin inhibitor) were added to resuspend the J2 pellet (28,29) and the suspension was incubated up to 4 hours at 37 °C with agitation for 10 seconds every 30 minutes. After concentration by centrifugation, the supernatant was filtered through a 0.22-μm Millipore (Millex-GV) membrane and concentrated with a 10,000-NMWL Immersible-CX Ultrafilter (Millipore, South San Francisco, CA). The concentrated extract was then diluted with three volumes of 2% SDS in 0.5 M Tris-HCL, pH 6.8, and concentrated to approximately 200 μl. The extract was stored at −70 °C.

Electrophoretic analyses: Polyacrylamide-SDS gel electrophoresis of CTAB-extracted SC was conducted according to Laemmli (14) using either 4-20% (w/v) gradient precast gels (Jule, New Haven, CT) or 6% or 12% homogenous gels. Following electrophoresis, the gel slab was silver-stained (23) or used unstained for Western blotting (39). The blots were blocked by incubation in 3% BSA for 2 hours at 25 °C and incubated in 1.25 μg/ml (1:2,000 of stock) alkaline phosphatase avidin (Avidin-AP, Calbiochem, San Diego, CA) for 1 hour at room temperature, followed by three washes with TBS. The blot then was developed in bromochloroindolyl phosphate-nitro blue tetrazolium (NBT-BCIP) (Sigma, St. Louis, MO) alkaline phosphatase substrate solution for 5 minutes. Blots of unlabeled J2 SC-treated with avidin-AP reagent served as controls for endogenous biotin. Western blots of radioiodinated J2 SC products were not treated with biotin-avidin reagents. Instead, autoradiographs were prepared by exposing blots to X-ray film for 7 days.

Glycosylated SC proteins from unlabeled J2 were detected by a modification of the method reported by Kaplan and Gottwald (12). Western blots of SC extracts were blocked with BSA as described above for biotin-avidin blotting. Blots were then incubated with agitation for 1 hour at 23 °C in biotinylated lectin (E-Y Laboratories, San Mateo, CA): Concanavalin A (1.0 μg/ml *Canavalia ensiformis* agglutinin), GS-1 (3 μg/ml *Griffonia simplicifolia* agglutinin), SBA (2 μg/ml *Glycine max* agglutinin), WGA (2 μg/ml *Triticum vulgarae* agglutinin), LPA (10 μg/ml *Limulus polyphemus* agglutinin), BPA (2 μg/ml *Bauhinia purpurea* agglutinin), DBA (4 μg/ml *Dolichos biflorus* agglutinin), UEA (2 μg/ml *Ulex europaeus* agglutinin), PNA (0.4 μg/ml *Arachis hypogaea* agglutinin) in HEPES-buffered saline (10 mM HEPES, 150 mM NaCl, 0.1 mM $CaCl_2.2H_2O$, 0.1 mM $MgCl_2.6H_2O$, 0.01 mM $MnCl_2.4H_2O$, pH 7.5). Biotinylated lectins that bound to the blotted proteins were visualized by incubation in 1.25 μg/ml alkaline phosphatase avidin in TBS, followed by NBT/BCIP development.

Immunology: Proteins extracted with CTAB were separated by SDS-PAGE on 6% (w/v) acrylamide slab gels. Proteins from a strip (one lane) of the gel were visualized by silver staining, and the strip was aligned with the remainder of unstained gel. The region of the unstained gel containing the SC_1 band was excised, and proteins were electro-eluted with constant current of 10 mA/Tube for 8 hours. After elution, the elution buffer (25 mM Tris-HCl, 192 mM Glycine, 0.1% SDS) was removed by dialysis against PBS. The resultant partially purified SC proteins were used as antigens for immunization. Rabbits were immunized subcutaneously (st) and intramuscularly (im) with a 2-3 μg suspension of SC proteins in saline mixed 1:1 with RIBI adjuvant (RIBI Immuno-Chem Research, Hamilton, MT), followed by three injections (st and im) with 0.5 μg of proteins in PBS 1:1 mixed with RIBI adjuvant at 3 to 4 week intervals. Serum was collected 3 weeks after the final immunization, and antibody was purified by ammonium sulfate precipitation and by Protein A-DEAE Affi-Gel Blue gel chromatography for the IgG fraction, according to the protocol provided by Bio-Rad (Bio-Rad Laboratories, Hercules, CA). Reactivity of the purified antibody with the nematode SC was determined by incubating live J2 in the antibody for 2 hours, washing them

three times with PBS, and incubating them in goat anti-rabbit antiserum-FITC (Sigma) for 1 hour. Preimmune serum and(or) elimination of the primary antibody were used as controls. After incubation, the treated nematodes were washed to remove unbound antibody conjugates and examined by fluorescence microscopy (21).

Proteins from CTAB extracts, separated by SDS-Page, were electroblotted to a nitrocellulose membrane (39) and then blocked in Blotto-Tween (5% nonfat dry milk (w/v) and 0.2% Tween 20 in 0.01 M, pH 7.2 PBS) and incubated in anti-SC IgG (1:100), containing 0.2% Tween 20, for at least 2 hours at 23 °C. Blotted proteins labeled with the anti-SC antibodies were detected by incubation in alkaline phosphatase-conjugated secondary anti-rabbit IgG (1.1 μg/ml, Sigma, St. Louis, MO) and then developed in NBT-BCIP.

RESULTS

The SC of *M. incognita* J2 covered the mid-body region in a continuous layer, approximately 5 nm thick, including the incisures of the transverse annulations (Fig. 1). It bound cationized ferritin and stained with ruthenium red (Fig. 1). Irregular patches or clumps associated with the SC also stained with ruthenium red, and similar-staining material often accumulated in the incisures (Fig. 1).

Electrophoretic profiles of proteins extracted or released from J2 labeled with either Biotin-NHS or ^{125}I showed similar patterns of labeling (Fig. 2). Both labeled the same principal protein (SC$_1$). Labeling with water-soluble biotin resulted in a greater number of labeled bands than labeling with biotin-NHS. The principal protein in all cases was greater than 250

FIG. 1. Electron micrographs of the surface coat (arrows) of *Meloidogyne incognita* infective juveniles. A) Nematode stained with ruthenium red. B) Nematode treated with cationized ferritin. Scale = 0.1 μm.
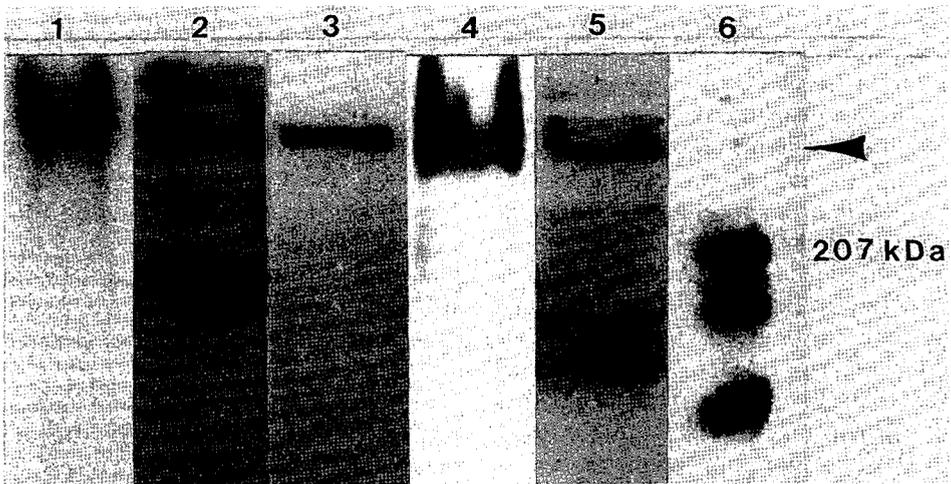
FIG. 2. Western blots of *Meloidogyne incognita* surface coat proteins separated on SDS gels and labeled with 1) ^{125}I (autoradiograph). 2) Biotin-X-NHS. 3) Biotin-NHS. 4) Biotin-X-NHS. Biotin was recovered from an aqueous incubate of biotinylated J2. 5) Concanavalin A. 6) Prestained standard proteins. SC_1 (arrow) = principal surface protein, approximately 250 kDa. Lanes 1, 2, 5, and 6 were separated on 4-20% gradient gels, lane 3 was separated on a 12% gel, and lane 4 was separated on a 6% gel.

kDa, based on comparison of its electrophoretic mobility with that of known proteins. Other, much fainter bands that labeled with water-soluble biotin ranged from 31 kDa to 179 kDa. The profiles of proteins labeled with biotin-avidin were not altered by the addition of a reducing agent, 2-mercaptoethanol, nor did the SC extract from non-biotinylated J2 react with avidin conjugates (data not shown).

.Release of ^{125}I-labeled surface components from the nematodes was demonstrated by incubating labeled J2 in water and assaying the incubation supernatant over a period of 20 hours. Radioactivity of the supernatant increased fourfold in the first hour and then decreased to the initial background level after 20 hours incubation (Table 1). The same trend was observed in a second trial with a different experimental design in which individual lots of ^{125}I-labeled J2 were incubated in separated tubes (data not shown). Because different experimental designs were used, the results could not be combined. Release of the SC also was demonstrated by detection of SC_1-biotin in aqueous incubates of labeled J2 (Fig. 2, lane 4).

Of the nine lectins tested, only Con-

canavalin A bound to SC_1 protein (Fig. 2, lane 5). It also bound to two smaller proteins, but because corresponding bands were not observed in blots of biotin-labeled SC proteins, these two were not considered to be associated with the surface coat.

Polyclonal antibodies raised to partially purified SC_1 bound to the J2 cuticular surface of *M. incognita* race 3 (Fig. 3A). On Western blots of CTAB extracts from *Meloidogyne* spp. J2, the purified IgG fraction of the antiserum reacted with proteins not only from *M. incognita* race 3, but also

TABLE 1. Release of ^{125}I from the surface of infective juveniles (J2) of *Meloidogyne incognita.*

Incubation time (hours)	CPM
0	23,854
1	77,993
2	66,100
4	59,905
8	57,045
16	46,704
20	24,517

CPM: Counts per minute (gamma emission) from a scintillation vial containing 20 cm^3 of water-miscible scintillation cocktail and 1.0 cm^3 of supernatant from ^{125}I-labeled *M. incognita* J2. Samples were drawn at intervals from a 2.0-cm^3 pool of labeled nematodes and, after sampling, the original volume of the pool was restored by addition of 1.0 cm^3 of sterile distilled water.
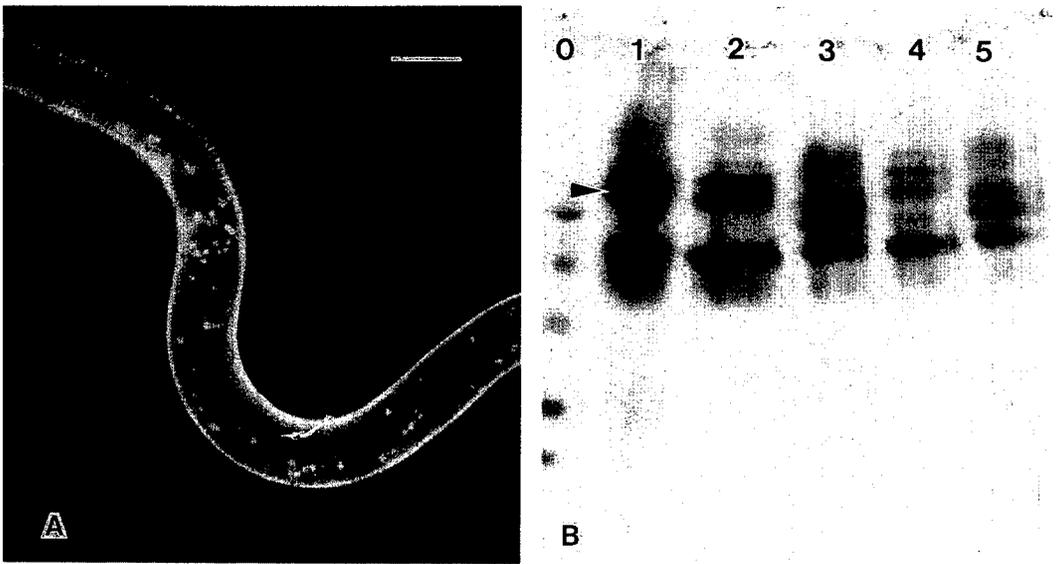
FIG. 3. A) Infective juvenile of *Meloidogyne incognita* treated with anti-SC$_1$ antibodies and FITC-conjugated goat, anti-rabbit immunoglobulins. Labeling of the surface coat is indicated by UV fluorescence. Scale = 10 μm. B) Western blot of CTAB extracts from unlabeled second-stage juveniles of different *Meloidogyne* spp., separated on a 4-20% SDS gel and probed with anti-SC antibodies against *M. incognita* race 3. (0) Prestained standards, top band = 207 kDa. (1) Extract from *M. incognita* race 3; (2) Extract from *M. incognita* race 2; (3) Extract from *M. incognita* race 1; (4) Extract from *Meloidogyne hapla* race 2: (5) Extract form *Meloidogyne arenaria* cytological race B. SC$_1$ (arrow).

with proteins from *M. incognita* races 1 and 2, *Meloidogyne hapla* race 2, and *Meloidogyne arenaria* cytological race B (Fig. 3B). A common band occurred among all *Meloidogyne* species, but because it was not labeled with either biotin or ^{125}I, it was not considered to have originated from the surface of the J2.

DISCUSSION

Two techniques were used to verify that proteins extracted from J2 of *M. incognita* with CTAB originated from the surface of the nematode. Both ^{125}I and biotin labeled the same principal band, SC$_1$, on Western blots and antibodies against SC$_1$ bound to the surface of J2.

It has been reported that the biotin-avidin system may not be suitable for identifying SC proteins of all nematodes. An endogenous avidin-binding protein has been identified in *C. elegans* that reduces the surface specificity of biotin label (Blaxter, pers. comm.), and similar nonspecific binding has been reported for *M. incognita*

race 3 (8). In the latter case, protein extracts were prepared from "body walls," which still had internal muscles attached and perhaps other internal contaminants as well. Because our CTAB extracts of intact, non-biotinylated *M. incognita* J2 did not react with avidin conjugates, it was assumed that endogenous avidin-binding substances do not occur on the surface of this nematode or, if they do, they were not extractable with CTAB under the conditions of this test. Water-soluble biotin labeled a greater number of CTAB-extractable proteins than did biotin with limited water solubility. Water-soluble markers may label internal nematode products that are extractable with CTAB but, because these proteins bound neither ^{125}I-Bolton Hunter reagent nor Concanavalin A, we have assumed that they are not associated with the SC of *M. incognita,* an assumption that may not be fully warranted without further investigation.

Surface proteins of many nematodes, both parasitic and free-living, have been examined by extrinsic radioiodination and

they typically represent a restricted set of molecules (17,25,27). In *Toxocara canis,* a nematode parasite of dogs, one surface glycoprotein (TES-120) is associated with the surface coat and is absent from other layers of the cuticle (24). Although we found no additional components labeled with ^{125}I in CTAB extracts, the existence of exposed proteins in the SC, which contain amino acid groups that do not react with ^{125}I-Bolton Hunter reagent, cannot be conclusively excluded.

The origin of the SC is controversial (35). However, it seems reasonable to hypothesize that it emanates either from the living hypodermis or from secretory glands associated with the excretory system (2) or nervous system, such as the amphids. Given a secretory origin, it could be expected that antibodies that bind to the SC will also bind to internal components of the nematode. In this study, we observed binding of anti-SC antibody to the body contents of ruptured nematodes, but it was not possible, in these nematodes, to localize the site of binding.

When Western blots of SC proteins were probed with Concanavalin A, only SC_1 (that also labeled with ^{125}I and biotin) was labeled (Fig. 2). This indicates that SC_1 is a glycosylated protein with exposed glucosyl or mannosyl residues. It is consistent with observations of other investigators, who have shown that Concanavalin A specifically binds to the surface of live J2 of *M. incognita* and can be displaced by treatment with competitive haptens (21,22,34). However, our evidence is not conclusive because competitive displacement assays were not conducted. Earlier work has shown that competitive displacement with single lectin-specific saccharides is often incomplete and difficult to interpret (21). Furthermore, the possibility that other bands in CTAB extracts contain sugar moieties, which may bind to lectins other than those tested, was not addressed.

Surface coat proteins found on animal-parasitic nematodes are thought, because of their lability, to play an active role in the evasion of host defense mechanisms (1,4,

10,11,25). In our investigation, SC proteins of *M. incognita* also were released from the nematode's surface when J2 were incubated in water. Early rapid release of ^{125}I from the surface of *M. incognita* may have resulted from an accelerated initial turn-over rate that reduced releasable SC proteins after 16 hours (Table 1). An important finding regarding the SC of animal-parasitic nematodes is that the process of shedding is an active one, inhibitable by metabolic arrest with azide or incubation at 4°C (10,32). This indicates that there may be a specific biochemical or enzymatic pathway that induces shedding. A similar process in plant-parasitic nematodes would enable them to adapt to soil biological environments and to deal with host resistance mechanisms that depend on interactions with the nematode's surface. Alternatively, SC proteins of plant-parasitic nematodes may provide the cues for host plant recognition and compatibility (8).

Antibodies to nematode antigens have been employed for differentiation of nematodes species, including those of *Meloidogyne* (7,9,26), and the application of serology to nematode diagnostics has been the subject of a recent review (6). But, a reliable serological system for distinguishing populations within species has not been advanced. Immunoblots (Fig. 3) revealed that SC proteins from different *Meloidogyne* spp. can be recognized by antibodies raised against individual species (*M. incognita* race 3), including various nontarget proteins that differ in molecular weight and antibody binding capacity. The SC immunogen obtained from J2 of *M. incognita* apparently induced a more general response in our experiment, and it may be possible to develop a serological system of diagnosis by using more specific antibodies raised against surface components.

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