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Cuticular Collagenous Proteins of Second-stage Juveniles and Adult Females of *Meloidogyne incognita*: Isolation and Partial Characterization[1]

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Abstract: Cuticles isolated from second-stage juveniles and adult females of *Meloidogyne incognita* were purified by treatment with 1% sodium dodecyl sulfate (SDS). The juvenile cuticle was composed of three zones differing in their solubility in β-mercaptoethanol (BME). Proteins in the cortical and median zones were partially soluble in BME, whereas the basal zone was the least soluble. The BME-soluble proteins from the juvenile cuticle were separated into 12 bands by SDS-polyacrylamide gel electrophoresis and characterized as collagenous proteins based on their sensitivity to collagenase and amino acid composition. The adult cuticle consisted of two zones which were dissolved extensively by BME. The basal zone was completely solubilized, leaving behind a network of fibers corresponding to the cortical zone. The BME-soluble proteins from the adult cuticle were separated by electrophoresis into nine bands one of which constituted > 55% of the total BME-soluble proteins. All bands were characterized as collagenous proteins. Collagenous proteins from juvenile cuticles also contained glycoproteins which were absent from the adult cuticles.

Key words: biochemistry, collagens, cuticle, electrophoresis, *Meloidogyne incognita*, root-knot nematode.

The cuticle of plant-parasitic nematodes is stucturally complex. It consists typically of an outer epicuticle, a cortical zone, a median zone, and an inner basal zone (6). The structure and thickness of the cuticle varies among nematode species, however, and frequently among developmental stages of the same species. Current knowledge of the biochemical composition of the nematode cuticle is based primarily on experiments with the animal-parasitic nematode *Ascaris lumbricoides* (15,20) and the free-living soil nematodes *Caenorhabditis elegans* (7) and *Panagrellus silusiae* (13). In these nematodes the cuticle has been shown to be composed of principally collagenous proteins extensively cross-linked by disulfide bonds. These proteins were found to be solubilized with the sulfhydryl agent, β-mercaptoethanol (BME). Cuticular collagenous proteins of plant-parasitic nematodes have not been studied, even though the ultrastucture of their cuticles has been investigated extensively (5,6). We describe here the isolation and partial characterization of cuticular collagenous proteins from preparasitic second-stage juveniles (J2) and parasitic adult females of *Meloidogyne incognita*.

Materials and Methods

Meloidogyne incognita (Kofoid and White) Chitwood was propagated on greenhouse-grown tomato plants. Eggs were extracted from galled roots as described by Hussey and Barker (11), and freshly hatched J2 were collected according to the procedure of Vrain (19). Parasitic adult females were isolated from infected tomato plants by macerating galled roots with pectinase according to Hussey (10).

Preparation of cuticles: Cuticles were purified essentially following the procedure of Cox et al. (7). Second-stage juveniles (5 ml) were suspended in 10 volumes of homogenization buffer (0.05 M tris-HCl containing 1 mM phenylmethylsulfonyl fluoride, pH 7.0) and sonicated in 20-second bursts with a Lab-line Ultratip sonicator (Lab-line Instruments, Melrose Park, Illinois) until all nematodes appeared broken when examined with a microscope. The homogenate was centrifuged for 10 minutes at 2,000 *g*, and the pellet was washed in the same buffer before being treated with five volumes of 0.1 M tris-HCl at pH 7.0, 1% SDS, 1 mM phenylmethylsulfonyl fluoride (STP buffer). After thorough washing, cuticles were suspended in two volumes of STP buffer, heated at 100 C in a boiling water bath for 2 minutes, incubated at ca. 22 C for 24 hours, and centrifuged at 5,000

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g for 10 minutes. The supernatant (STP-soluble proteins) was stored at −20 C until used. The pellet was washed in STP buffer at ca. 22 C three more times before proceeding further.

Adult female cuticles (1–2 ml) were prepared by homogenizing the nematodes in a Dounce homogenizer (20 strokes) in the same buffer used for J2 and processing the homogenate in the same manner as J2 cuticles.

Isolation of collagens: BME-soluble proteins were prepared according to Cox et al. (7). Briefly, STP-extracted cuticles were suspended in STP buffer containing 5% BME and heated at 100 C in a boiling water bath for 2 minutes. Cuticles were then incubated at ca. 22 C for 24 hours and centrifuged at 5,000 *g* for 10 minutes. The supernatant (BME-soluble proteins) was stored at −20 C until used. Protein concentrations were determined after Lowry et al. (14).

Enzyme digestions: BME-soluble proteins (100 μg) in STP buffer were precipitated in 90% acetone, air dried, suspended in tris buffer (0.05 M tris-HCl, 0.1 M NaCl, 5.0 mM calcium chloride, 10 mM N-ethylmaleimide, pH 7.2), and incubated at 37 C for 12 hours with and without 100 units of collagenase (*Clostridium*, Form III, Advanced Biofactures Corp., Lynbrook, New York) or 10 μg of Proteinase K (Sigma Chemical Co., St. Louis, Missouri). Control incubations under identical conditions were performed with bovine serum albumin and human placental collagen (Sigma Chemical Co.). At the end of the reaction time, the reaction mixture was added to STP buffer and heated at 100 C for 5 minutes. Material equivalent to 25 μg protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 7% gels and silver stained. Human placental collagen and bovine serum albumin served as positive and negative controls for collagenase digestion.

Amino acid analysis: BME-insoluble cuticles and acetone-precipated, air dried, BME-soluble samples were hydrolyzed in 6 N HCl and the amino acid compositions determined by standard ion-exchange chromatographic methods by Genetic Design, Inc. (Watertown, Massachusetts). Cysteine was determined as cysteic acid.

Electron microscopy: STP buffer-treated cuticles, and STP buffer-extracted plus BME-extracted cuticles, were washed with deionized distilled water and fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) at 4 C overnight. After rinsing with buffer the samples were postfixed in cold 1% OsO_4 in 0.2 M cacodylate buffer (pH 7.2) for 1 hour. The cuticles were dehydrated in a cold graded ethanol series and infiltrated with Spurr's resin. Thin sections were stained with saturated uranyl acetate and lead citrate and viewed in a Philips EM400 at 100 keV.

Electrophoresis: Discontinuous SDS–PAGE was performed according to Laemmli (12). Staining was with 0.1% Coomassie Blue R-250 in 40% methanol plus 7.5% acetic acid. For detecting glycoproteins, gels were fixed in 7.5% acetic acid for 1 hour and treated with 0.2% periodic acid at 4 C for 1 hour before incubating in Schiff base overnight (1). Silver staining was according to Morrissey et al. (17).

Results

Cuticles: Treatment of J2 and adult female cuticles with STP buffer yielded preparations free of underlying hypodermal and muscle tissues and extraneous contaminants (Figs. 1a, 2a). The J2 cuticle was seen as a three-zoned structure consisting of an outer electron-dense cortical zone, a clear median zone with struts, and a striated basal fiber zone (Fig. 1b). The cortical zone was covered by a triple-layered epicuticle.

The adult female cuticle had two primary zones—cortical and basal (Fig. 2b). The cortical zone was covered by a thin electron-dense epicuticle. The cortical zone, which was not clearly distinguishable, varied in thickness and contained a network of thin fibers apparently permeated by the basal zone. The thick basal zone predominated in the female cuticle.

Isolation of collagenous proteins: The internal structure of J2 and adult female cuticles was altered by extracting BME-soluble proteins. In J2 cuticles extraction with BME resulted in a frayed epicuticle (Fig. 1d) with a pitted appearance at low magnification (Fig. 1c). The median zone was considerably less electron dense than the epicuticle, indicating its partial solubility in BME, and the struts were completely

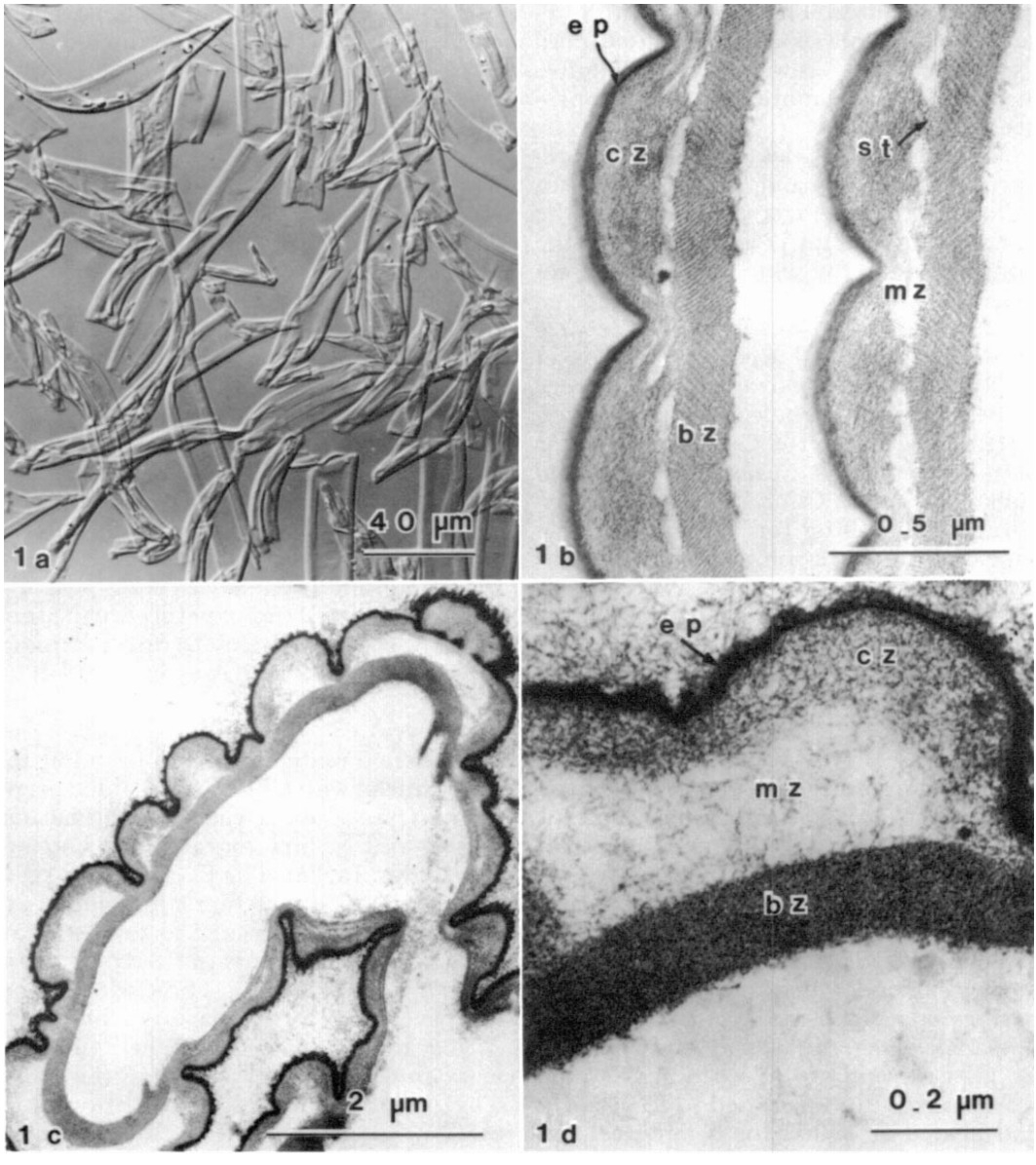

FIG. 1. *Meloidogyne incognita* second-stage juvenile cuticles before and after treatment with β-mercaptoethanol (BME). a) Differential interference contrast micrograph of sodium dodecyl sulfate (SDS)-purified cuticles. b) Longitudinal transmission electron micrograph of SDS-purified cuticles showing three-zoned structure. Epicuticle (ep) covers the corticle zone (cz). Median zone (mz) is transversed by columnar structures called struts (st) which join the cortical zone to a striated basal zone (bz). c, d) Longitudinal transmission electron micrographs of purified cuticles after treatment with BME showing differential solubility of zones in the disulfide agent.

dissolved. The basal zone of the juvenile cuticle was dissolved the least. The striated appearance of the basal zone was diminished, and this zone seemed to shrink in that the cortical zone appeared buckled (Fig. 1c).

In contrast, adult female cuticles were solubilized extensively by BME. Of the two primary zones, the thick basal zone was completely soluble. Fibrous material left from the cortical zone remained attached to a now less electron-dense epicuticle (Fig. 2c).

Electrophoretic characterization of collage-

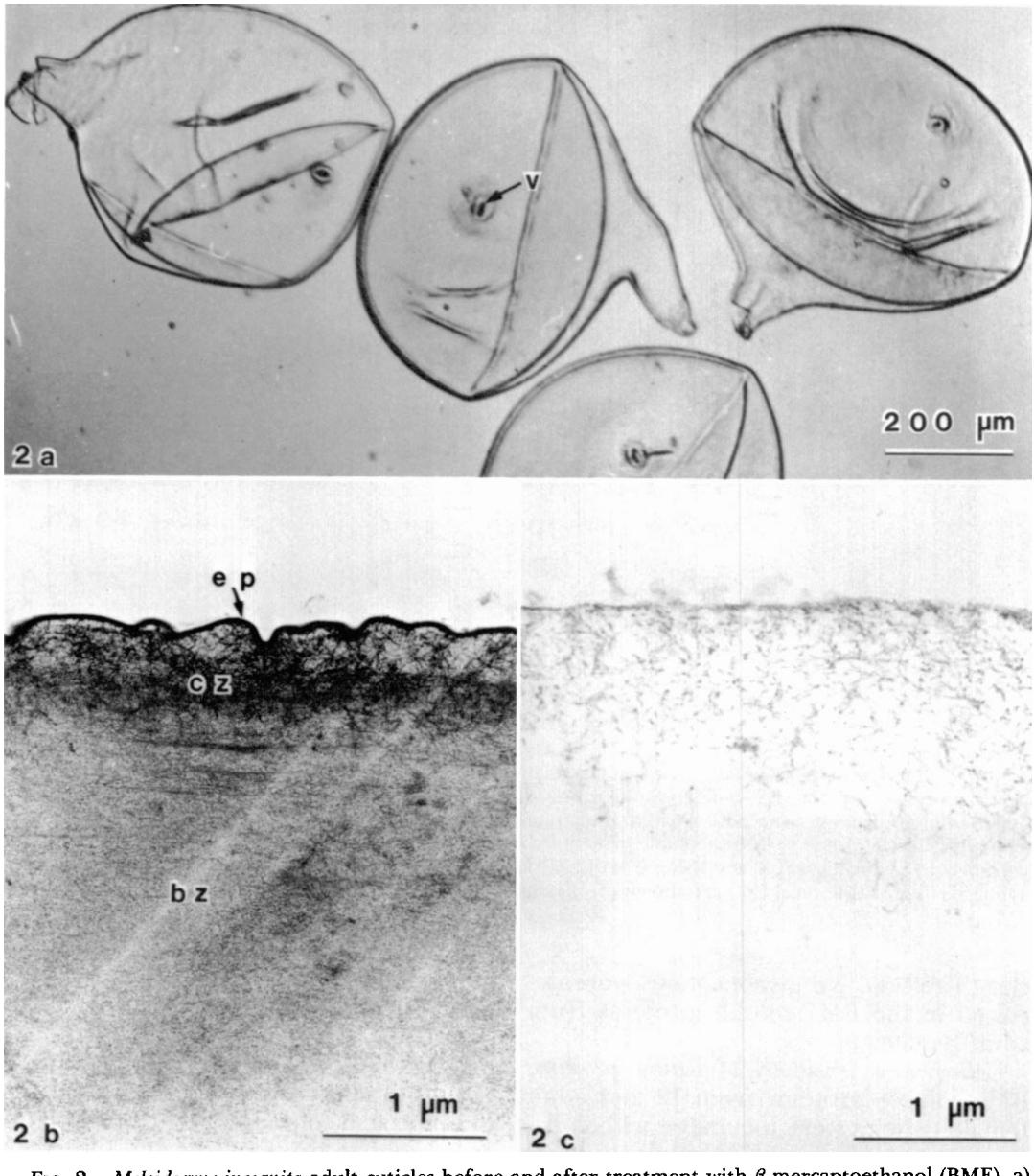

FIG. 2. *Meloidogyne incognita* adult cuticles before and after treatment with β-mercaptoethanol (BME). a) Differential interference contrast micrograph of sodium dodecyl sulfate (SDS)-purified cuticles showing vulva (v). b) Transmission electron micrograph of SDS-purified cuticle showing two-zoned structure. Cortical zone (cz) is covered by the epicuticle (ep). Basal zone (bz) is very thick and not clearly separated from the cortical zone. c) Transmission electron micrograph showing cuticle material remaining after treatment with BME.

nous proteins: Cuticular proteins solubilized by STP buffer and STP plus 5% BME were analyzed by SDS–PAGE. BME-soluble proteins from preparasitic J2 cuticles separated into 12 bands, those from adult female cuticles into nine bands (Fig. 3). One protein from the adult cuticles (fp5) constituted approximately 55% of the total BME-soluble proteins. All bands exhibited metachromatic staining (pink) with Coomassie Blue R-250. Molecular weights of the individual proteins are presented in Table 1. STP soluble protein (Fig. 3) did not exhibit metachromatic staining.

BME-soluble proteins from J2 and adult female cuticles were also stained for glycoproteins. There are seven glycoproteins in the BME-soluble fraction from J2 cuti-

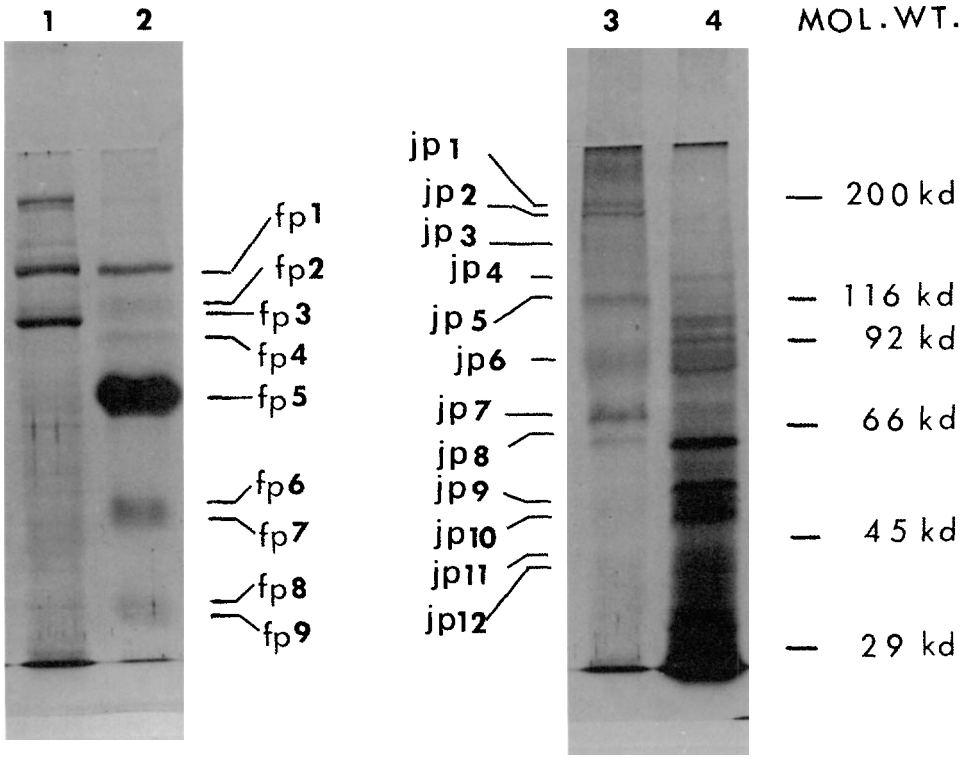

FIG. 3. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis of *Meloidogyne incognita* soluble cuticle proteins. Lane 1) SDS–buffer-soluble proteins from adult female cuticles. Lane 2) β-mercaptoethanol (BME)-soluble proteins from STP-treated adult female cuticles. Lane 3) BME-soluble proteins from STP-treated J2 cuticles. Lane 4) Buffer-soluble proteins from J2 cuticles. BME-soluble proteins from adult females are labeled Fp1 through Fp9, and those from J2 are labeled Jp1 through Jp12. Positions of molecular weight standards (Sigma Chemical Co.) are shown on the right. Each lane contained 50 μg of protein.

cles (Table 1). No glycoproteins were detected in the BME-soluble proteins from adult female cuticles.

Collagenase sensitivity of cuticle proteins: BME-soluble proteins from J2 and adult female cuticles were incubated with collagenase, and the digestion mixtures were analyzed by SDS–PAGE. Human placental collagen proteins and bovine serum albumin were also subjected to similar treatments as positive and negative controls, respectively. The BME-soluble proteins from adult female cuticles were completely digested by collagenase (Fig. 4, lanes 6 and 8). All but one of the BME-soluble proteins from J2 cuticles were completely digested (Fig. 4, lanes 9 and 11). This one protein (molecular weight, 70,000) was only partially digested. The collagenase employed in these studies was free of nonspecific proteolytic activity, as shown by its inability to digest bovine serum albumin. However, human placental collagen proteins were almost completely digested (Fig. 4).

Amino acid composition: J2 and adult female cuticles were distinct from each other (Table 2) and typical of collagen profiles in amino acid compositions of BME-soluble and insoluble proteins. Several significant differences occurred in the amino acid profiles of the BME-soluble proteins of J2 and adult female cuticles. J2 cuticles contained considerably more glycine residues (40%) than the adult female cuticles (31%). In addition to the quantities of several other amino acid residues being greater in J2 cuticles, a trace amount of hydroxylysine was detected in J2 cuticles but not in the adult female cuticles. Concentrations of other amino acid residues were significantly higher in adult female than in J2 fractions. The adult female cuticles contained more glutamic acid, aspartic acid, and serine than did the J2 soluble proteins. The amino acid

TABLE 1. Apparent molecular weights of collagenous proteins from *Meloidogyne incognita* juvenile and adult female cuticles.

Juvenile cuticle		Adult female cuticle	
Protein	Mol. wt*	Protein†	Mol. wt.*
Jp1	186,000	Fp1	140,000
Jp2‡	180,000	Fp2	117,000
Jp3‡	155,000	Fp3	116,000
Jp4‡	133,000	Fp4	103,000
Jp5	123,000	Fp5	76,000
Jp6	93,000	Fp6	50,000
Jp7	70,000	Fp7	48,000
Jp8‡	67,000	Fp8	34,000
Jp9‡	49,000	Fp9	32,000
Jp10‡	47,000		
Jp11	41,000		
Jp12‡	39,000		

* Molecular weights were averaged from two separate determinations on 7% SDS–polyacrylamide gels.

† No glycoproteins were detected in adult female β-mercaptoethanol-soluble proteins.

‡ May be glycosylated.

profiles of the BME-insoluble cuticle proteins from J2 and adult female cuticles were similar (except for hydroxylysine) to the soluble protein fractions but differed quantitatively.

DISCUSSION

Ultrastructural studies have shown that nematodes can exhibit stage-specific differences in cuticle structure (5,6). A systematic biochemical analysis of cuticular components at each developmental stage has been carried out for only two nematodes. Cox et al. (8) have shown that the expression pattern of cuticular collagens differed for each developmental stage of the free-living nematode *C. elegans*. On the other hand, Leushner et al. (13) reported a quantitative rather than qualitative variation in the collagenous proteins of the free-living nematode, *P. silusiae*. Comparative

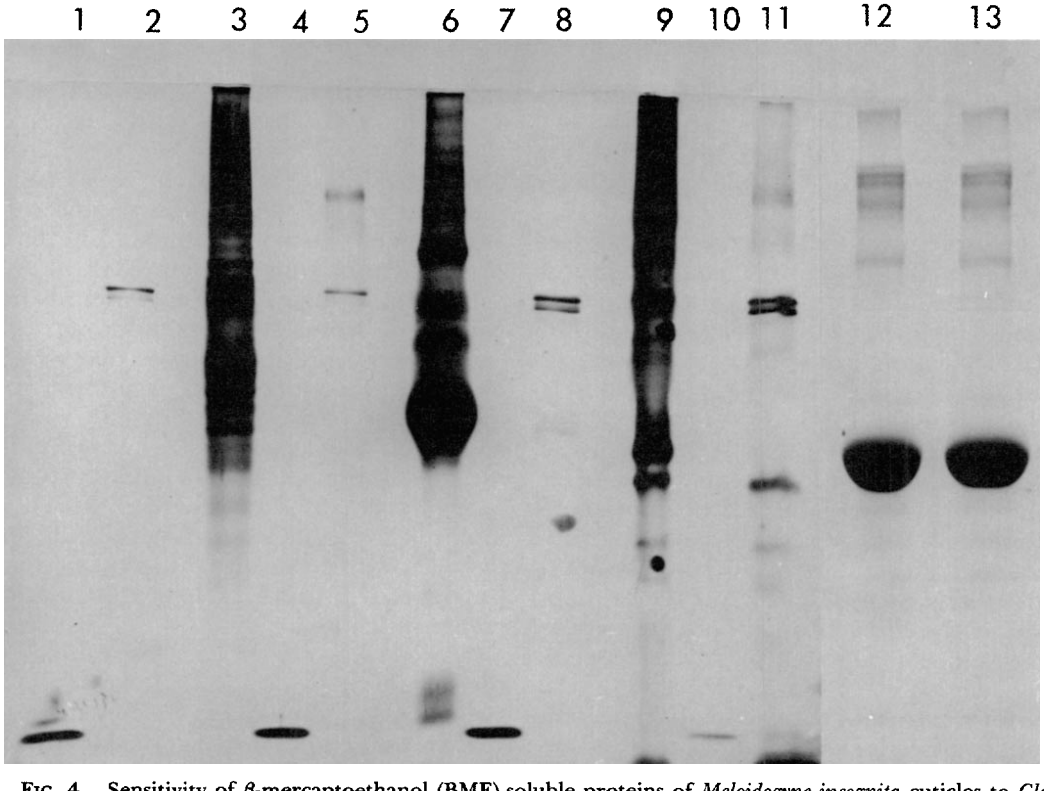

FIG. 4. Sensitivity of β-mercaptoethanol (BME)-soluble proteins of *Meloidogyne incognita* cuticles to *Clostridium* collagenase and proteinase K. Lane 1) Proteinase K (2 μg). Lane 2) *Clostridium* collagenase (2.5 units). Lane 3) Human placental collagen. Lane 4) Human placental collagen and proteinase K. Lane 5) Human placental collagen and collagenase. Lane 6) *M. incognita* adult female BME-soluble proteins. Lane 7) Adult female BME-soluble protein plus proteinase K. Lane 8) Adult female BME-soluble proteins plus collagenase. Lane 9) J2 BME-soluble proteins. Lane 10) J2 BME-soluble proteins plus proteinase K. Lane 11) J2 BME-soluble proteins plus collagenase. Lane 12) Bovine serum albumin. Lane 13) Bovine serum albumin plus collagenase.

TABLE 2. Amino acid composition of β-mercaptoethanol (BME)-soluble and BME-insoluble fractions from *Meloidogyne incognita* juvenile and adult female cuticles.* Residues per 1,000 total amino acids.

Amino acid	Juvenile		Adult female	
	Soluble	Insoluble	Soluble	Insoluble
Aspartic acid†	23.66	48.32	40.28	51.31
Threonine	26.40	26.37	15.35	27.90
Serine	15.43	46.84	38.70	52.02
Glutamic acid†	35.72	83.24	125.42	93.15
Proline	150.51	118.52	166.38	119.76
Hydroxyproline	49.76	46.82	59.18	39.62
Glycine	378.80	416.38	292.63	319.86
Alanine	123.10	91.32	106.14	110.20
Cysteine‡	23.64	Not determined	37.48	Not determined
Valine	28.20	21.90	24.05	33.66
Methionine	5.15	11.34	0	7.00
Isoleucine	22.10	15.87	13.71	21.50
Leucine	23.18	16.28	12.46	25.78
Tyrosine	18.37	7.28	12.26	22.92
Phenylalanine	13.60	0	0	18.37
Lysine	33.74	26.22	32.30	30.68
Hydroxylysine	Trace	0	0	0
Histidine	13.38	8.80	11.16	9.74
Arginine	15.26	14.47	11.77	16.49

* Based on 48 hours hydrolysis. Analysis was performed by Genetic Design, Inc./Sequemat, Inc., Watertown, Mass.
† Amidic forms included.
‡ Determined as cysteic acid.

biochemical data for other nematode cuticles do not exist. This is the first report on the biochemical and ultrastructural characterization of cuticular collagenous proteins of a plant-parasitic nematode.

Treatment with STP had little effect on the internal structure of *M. incognita* J2 and adult female cuticles. The ultrastructure of these cuticles remained very similar to in situ electron micrographs of cuticles of J2 and adult females of *M. javanica*, a closely related species (3,4). However, a slight change in the appearance of the epicuticle occurred after STP treatment. Proteins solubilized during STP treatment are considered noncollagenous, because the subsequent cuticle ultrastructure looks unperturbed and the solubilized proteins do not exhibit the metachromatic staining characteristic of collagens (16). Also, Cox et al. (7) have suggested that proteins present in the STP extract of cuticles might represent solubilized hypodermal and muscle zones that underlie the nematode cuticle.

Treatment of STP-washed cuticles of J2 and adult females with BME resulted in drastic alterations in the internal structure of the cuticle. Each zone of the cuticle was affected by BME to a different extent, indicating that the degree of disulfide linkages varies from zone to zone. It appears that most of the proteins solubilized from J2 cuticles by BME were extracted from the cortical and the median zones, since the structure of these zones seems to be altered more than the others. The basal zone was mostly insoluble in BME.

It is not known if all the protein bands represent unique collagenous protein chains. Some of the high molecular weight bands may be unreduced or unreducible aggregates of lower molecular weight species on these gels (18). The molecular weight of the J2 BME-soluble proteins ranged from 39,000 to 186,000. These molecular weights are considered only tentative, however, because the associated carbohydrate renders accurate estimation rather difficult.

The *M. incognita* J2 cuticle is similar to the cuticle of *C. elegans* dauer juvenile in structure and solubility in BME. Under adverse environmental conditions, the *C. elegans* J2 develops into a survival stage called the dauer juvenile. In this state, the animal does not feed and can survive for exceptionally long periods of time. The cuticle of the dauer stage of *C. elegans* is more resistant to BME than are the other life stages of this nematode. The J2 is the prin-

cipal juvenile survival stage of *M. incognita*. It is the only juvenile stage that is free living in the soil, and it must locate roots of a susceptible plant to continue its life cycle. Because the J2 may encounter adverse environmental conditions while migrating in the soil, it needs an exoskeleton that can withstand fluctuating conditions. The striated basal zone of the *Meloidogyne* J2 cuticle (4,5) is structurally similar to the basal zone of the *C. elegans* dauer juvenile (8). The striated structure is thought to contribute to the chemical resistance of the basal zone; it is characteristic of nematode life stages that are exposed to fluctuating environments (5). After the *Meloidogyne* J2 locates and penetrates a host root and becomes parasitic, the internal structure of its cuticle begins to change (4). The principal change occurs in the basal zone where the striations disappear and the cuticle begins to look like an adult female cuticle. This pattern resembles that of the *C. elegans* dauer juvenile, which reverts to a normal developmental cycle (J4 and adult stages) upon encountering favorable environmental conditions; its internal cuticle structure also changes concomitantly.

Whereas *C. elegans* females remain vermiform throughout their life cycle, soon after a *Meloidogyne* J2 commences its parasitic mode of life inside a root, it grows until it becomes a saccate and sedentary adult female. This increase in size and drastic alteration in shape is accompanied by biochemical and ultrastructural changes in the cuticle. As we have shown here, the biochemical composition of the adult female cuticle differs significantly from the J2 cuticle. Most notably, one protein with a molecular weight of 76,000 constitutes > 55% of the total BME-soluble proteins from the adult female cuticle. Experiments are in progress to purify this protein and produce antibodies to it for use in determining its location in the adult female cuticle. In addition, the adult female cuticle was considerably more susceptible than J2 cuticles to BME, with the basal zone and part of the cortical zone of the adult female cuticle being completely soluble in the sulfhydryl agent. It is reasonable to assume that most of the proteins solubilized by BME from the adult female cuticle are from the basal zone. In this respect, the *M. incognita* cuticle is similar to the *C. elegans* cuticle in all of its developmental stages except the dauer juvenile. In *C. elegans* J4 and adult cuticles, the basal zone is completely dissolved by BME leaving behind only the epicuticle and traces of the cortical zone (8). A major portion of the basal zone of the J1 cuticle is dissolved by BME, and J2 and J3 are also said to be very similar to J4 and adult stages (8). These observations lend support to Bird's (6) suggestion based on ultrastructural studies that the cuticle structure of nematodes is an adaptation in response to the environmental conditions to which the animal is exposed.

The collagenous nature of BME-soluble proteins from *M. incognita* J2 and adult female cuticles was confirmed by their susceptibility to digestion by *Clostridium* collagenase and their amino acid compositions. BME-soluble proteins were extensively degraded by this enzyme. Partial resistance of one J2 cuticle protein might be caused by associated carbohydrates, as several BME-soluble proteins were identified as glycoproteins. Moreover, Goldstein and Adams (9) have shown that certain annelid collagens are incompletely digested by *Clostridium* collagenase. The amino acid profiles of BME-soluble proteins from J2 and adult female cuticles are similar to those of standard collagens (2) with high concentrations of glycine, alanine, cysteine, and proline. Of the four fractions analyzed, only J2 BME-soluble proteins contained trace amounts of hydroxylysine. Only the *C. elegans* dauer juveniles contained traces of hydroxylysine (8), indicating another similarity between its cuticle and the cuticle of *M. incognita* J2.

The amino acid profiles of BME-insoluble proteins of both stages of *M. incognita* are distinct from each other and from their respective BME-soluble counterparts; they also resemble profiles typical of collagens. It is assumed that the BME-insoluble cuticles are composed of a different type of collagenous proteins crosslinked by nondisulfide covalent bonds. Experiments are underway to study the susceptibility of these BME-resistant cuticles to different collagenases.

The data reported here show that the cuticle of *M. incognita* contains collagenous proteins that are interlinked to each other

and to other cuticular components through disulfide bonds. A complete biochemical analysis of the cuticular components of different life stages may provide information that will enable the development of new approaches to control this important plant-parasitic nematode.

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