Journal of Nematology 21(4):477–482. 1989.

Composition of High Molecular Weight Excretions–Secretions from Infective Larvae of *Meloidogyne javanica*[1]

A. Bacic,[2] Z. Felton,[2] and A. F. Bird[3]

Abstract: Infective larvae (J2) of *Meloidogyne javanica* were incubated in distilled water for up to 14 days, and their high molecular weight (> 1,000 daltons) excretions–secretions (ES) were isolated and partially characterized. The ES consisted of a mixture of proteins, glycoproteins, and proteoglycans or polysaccharides as revealed by differential staining on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and compositional analysis. Carbohydrate, with approximately equal amounts of neutral monosaccharides and hexosamines, was the major constituent of the ES, with only low levels of protein detected. Acidic sugar residues, including sialic acids, were not detected.

Key words: carbohydrate composition, excretion–secretion, infective larval stage, *Meloidogyne javanica*, root-knot nematode.

Plant-parasitic nematodes secrete numerous organic substances. Some of these are recognized as being important in infection of plant roots and induction of giant cell formation by the second-stage infective larvae (J2) of *Meloidogyne* spp. as well as the maintenance of the giant cells by the adult females (4). More recently it has been suggested that the "excretory" system of *Caenorhabditis elegans* (32) and that of *Anguina agrostis* (6) may be the site of secretion of the surface coat (glycocalyx) glycoconjugates which may act as a lubricant to assist movement through the soil. Surface coats may also act as recognition sites for the attachment of host-specific parasitic micro-organisms. For example, the stage-specific, and species-specific attachment of bacterial endospores of *Pasteuria penetrans* to *M. javanica* J2 may be mediated in part by an interaction involving the surface coat glycosyl residues (7).

Previous studies of the excretions–secretions of plant-parasitic nematodes have focused on nitrogeneous compounds (1,8,9,30,35,38,39) and low molecular weight carbohydrates (39). In this paper we report on the isolation and partial structural characterization of the high molecular weight (> 1,000 daltons) excretions–secretions (ES) of *M. javanica* J2.

Materials and Methods

Nematodes: Meloidogyne javanica J2 were obtained from egg masses dissected from the roots of greenhouse-grown tomato (*Lycopersicon esculentum*) plants. The egg masses were sterilized in 0.5% (v/v) chlorhexidine gluconate (Hibitane) for 20 minutes, washed three times in sterile distilled water (SDW), and poured into sterile petri dishes, or the egg masses were separated from their gelatinous matrix by treatment with 0.5% (w/v) sodium hypochlorite for 1.5 minutes, washing into a 30-μm-pore sieve, decantation into a large volume of SDW, and removal from the remainder of the matrix by passage through a 76-μm-pore sieve (10). ES, collected (as outlined in the next paragraph) from J2 derived from egg masses sterilized by either procedure, was combined prior to analysis.

Collection of excretions–secretions: The eggs were hatched in SDW at 23 C. Egg mass and larval exudate from 10^5–10^6 nematodes was collected in SDW at 23 C at 2–7 day intervals over a 14-day period. These exudates were examined for bacterial contamination by direct observation under an interference contrast microscope and by plating aliquots onto 1% (w/v) peptone–

Received for publication 30 September 1988.

[1] This work was supported in part by a joint University of Melbourne–C.S.I.R.O. grant.

[2] Plant Cell Biology Research Centre, School of Botany, The University of Melbourne, Parkville, Victoria 3052, Australia.

[3] C.S.I.R.O. Division of Soils, Private Mail Bag 2, PO Glen Osmond, South Australia 5062, Australia.

We thank Moira Robinson and Jodie Weinhandl for technical assistance.

TABLE 1. Composition of high molecular weight excretions–secretions of *Meloidogyne javanica*.

Constituent	% w/w
Neutral carbohydrate	37.0
Fucose	9.9
Xylose	0.7
Mannose	0.7
Galactose	16.4
Glucose	9.3
Uronic acids	n.d.†
Hexosamines	33.2
Glucosamine	30.2
Galactosamine	3.0
Sulfate	
N-	5.4
O-	10.1
Protein	3.6
Phosphate	0.2

† Not detected.

glucose agar plates incubated at 37 C for 24–72 hours before passage through a 0.45-μm-pore filter and collection in sterile bottles. Exudates contaminated with bacteria were discarded. In addition, the condition of the J2 at the end of the 14-day incubation period was assessed microscopically. Apart from depleted lipid reserves, all the nematodes remained viable and in an apparently healthy condition as judged by their ability to infect plant roots (Bird, unpubl.).

The exudates were then concentrated ca. 10-fold (i.e., to 1–2 ml) at 4 C in a Diaflo apparatus (Amicon Corp., Danvers, MA) using a YM-2 filter (molecular weight cut-off 1,000 daltons). After concentration, the samples were dialyzed exhaustively at 4 C on the same membrane filter, and the high molecular weight ES were lyophilized for analyses.

Analytical techniques: *N*-sulfated hexosamines present difficulties on hydrolysis for constituent sugar analysis, as the sulfate group is released rapidly giving rise to an extremely stable glycosidic linkage (3). In order to overcome this problem, the ES was first desulfated and then *N*-acetylated as described by Nagasawa and Inoue (31) prior to monosaccharide analyses. Monosaccharides were determined as their per-*O*-trimethylsilyl methylglycosides following methanolysis (1 M methanolic HCl, 16 hours, 80 C) (16). The trimethylsilylated derivatives, in *n*-hexane, were separated and quantified by gas chromatography (GC) and identified by GC-mass spectrometry (GC-MS) (Finnigan MAT 1020B, San Jose, CA) using a CPSil-5 wall-coated fused silica column (25 m × 0.32 mm i.d., Chrompack, Middelburg, The Netherlands). For GC, the oven temperature was held at 50 C for 30 seconds, then heated to 190 C at 70 C minute^{-1}, and then from 190 C to 320 C at 6 C minute^{-1}. For GC-MS, the GC oven was held for 2 minutes at 110 C and then increased from 110 C to 320 C at 6 C minute^{-1}. The injector port was held at 150 C. Compounds were detected by electron-impact ionization in the MS using the total ion current by scanning from m/z 70–700 in 0.5 seconds.

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12.5% polyacrylamide) was performed (25) on the native ES fraction. Gels were stained for protein with Coomassie Blue and silver (Bio-Rad, Richmond, CA) and for carbohydrate with periodic acid-Schiff reagent (PAS) (Pharmacia, Uppsala, Sweden).

General methods: Total carbohydrate was determined colorimetrically by the phenol-sulfuric acid method (18), using glucose as the standard. Total protein was determined with a bovine serum albumin standard by the Bio-Rad microassay method (14). Uronic acids were estimated colorimetrically by the carbazole method of Dische (17) as modified by Galambos (21) using polygalacturonic acid as a standard. Hexosamines (glucosamine and galactosamine) and *N*-sulfate and *O*-sulfate were estimated colorimetrically (15). Phosphate was determined by the method of Bartlett (2).

RESULTS

The amount of ES collected varied from 1.0 to 1.5 ng per nematode per day on a dry weight basis. The ES was composed

primarily of carbohydrate (ca. 70%) and a small amount of protein (Table 1). In addition, sulfate, both *N*- substituted and *O*-substituted, was present in significant amounts. Although the levels of protein in the ES remained relatively constant in different batches, there was some variability in the relative proportion of hexosamine to neutral carbohydrate and in the degree of *N*- and *O*-sulfation (data not shown). There were approximately equal proportions of neutral sugars and hexosamines in the ES carbohydrate. Uronic acids were not detected. The hexosamine was primarily glucosamine with smaller amounts of galactosamine (Table 1). Whether the hexosamines are present as their *N*-acetylated derivatives was not determined, although the presence of *N*-sulfate indicates some are present as the *N*-substituted (sulfate) derivatives (Table 1). Fucose, galactose, and glucose were the major monosaccharides, together with small amounts of xylose and mannose (Table 1). Sialic acid was not detected in the ES of the desulfated *N*-acetylated or the native fraction.

One major and four minor protein bands were detected by silver and Coomassie Blue staining after SDS-PAGE of native ES (Fig. 1a). The major protein band at ca. 100 kilodaltons also stained for carbohydrate by the periodic acid-Schiff stain (Fig. 1b), suggesting it is a glycoprotein. In addition, the carbohydrate staining revealed the presence of a high molecular weight smear (> 100 kilodaltons), which also stained for protein.

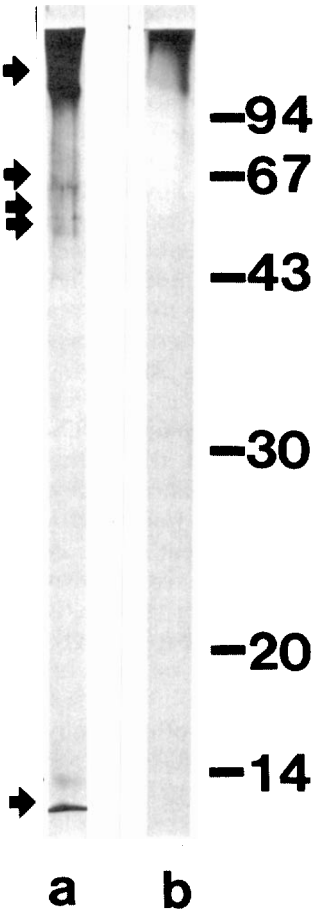

FIG. 1. One-dimensional sodium dodecyl sulfate-polyacrylamide gels of native ES of the infective larval stage (J2) of *Meloidogyne javanica*. a) Stained with Coomassie Blue followed by silver. b) Stained with periodic acid-Schiff reagent. Numbers correspond to commercial molecular weight markers (in kilodaltons).

DISCUSSION

The carbohydrate moiety of cell surface glycoconjugates (glycolipids, glycoproteins, proteoglycans, and polysaccharides) is increasingly being implicated in recognition phenomena, including cell–cell and host–parasite interactions (19,33,34,41). The surface of nematode cuticles, particularly the origin and chemistry of the surface coat (glycocalyx) and its relationship to the epicuticle, is poorly understood (5,40). In addition, the surface chemistry of nematodes is variable both between and within species as demonstrated by the species-specific and stage-specific adhesion of micro-organisms (11,12,37). Lectin studies on a range of nematode species have demonstrated the presence of sugar residues—including glucose or mannose, galactose or *N*-acetylgalactosamine, *N*-acetylglucosamine, and sialic acid—on the cuticle surface (6,7,13,20,24,26,36,41,42). Experimental evidence from two groups on two different genera, *Caenorhabditis* (32) and *Meloidogyne* (6), suggests that the surface glycocalyx may be secreted via the ex-

cretory pore. It seemed likely, therefore, that the incubation medium in which the nematodes were cultured would also contain some of this surface coat material and that this may be a useful means of collecting sufficient material to study its chemistry. We were aware, however, that this also would contain any exudates originating from the other cuticular orifices including the mouth, anus, amphids, and phasmids of the J2 in addition to some material that may have been released and (or) solubilized from the egg shells and their gelatinous matrix.

Our results are the first preliminary constituent analysis of the high molecular weight (> 1,000 daltons) ES of a plant-parasitic nematode. Carbohydrate is clearly a major component of the ES fraction. Carbohydrate can exist in polymeric form as polysaccharide or, alternatively, covalently attached to protein (glycoproteins and proteoglycans) or lipid (glycolipids). The coincident staining of a sharp, clearly defined band for both carbohydrate and protein on SDS-PAGE indicates that the major protein is a glycoprotein. In animal glycoproteins, the glycan moiety is covalently *N*-linked or *O*-linked to protein (22). *N*-linked glycoproteins contain fucose, mannose, and *N*-acetylglucosamine in their core structure. The absence of significant levels of mannose suggests that *N*-linked glycoproteins, if present, are minor constituents. In contrast, *O*-linked glycoproteins contain *N*-acetylgalactosamine linked to serine, and galactose, *N*-acetylglucosamine, sialic acid, and fucose are common monosaccharides. The presence of low levels of *N*-acetylgalactosamine would suggest only low levels of *O*-linked glycoproteins; however, it is conceivable that long glycan chains of *N*-acetylglucosamine, galactose, and fucose are present.

These (glyco) protein bands differ somewhat from those in stylet exudate from adult females of *M. incognita* (38), indicating either species-specific or stage-specific secretion. Although proteases have not been reported in the excretions–secretions of plant-parasitic nematodes, if present, they would be expected to modify the proteins in the ES during these long collection periods (2–7 days). This may explain the apparent variance between the intense staining for protein in various exudates observed during microscopic studies (4,35,38) and the low levels of protein detected (Table 1).

The high molecular weight (> 100 kilodaltons) smear, which stains for both carbohydrate and protein, is strongly suggestive of a proteoglycan. These are characteristically polydisperse molecules. The animal proteoglycans contain polysaccharide chains covalently attached to a protein core. All animal proteoglycans, except for keratan sulfate, contain a repeating disaccharide of a uronic acid and a hexosamine, variously sulfated (both *N* and *O*) (22). The absence of uronic acids in our analyses precludes these classes of animal proteoglycans; however, keratan sulfate differs from the other animal proteoglycans in lacking uronic acid residues. It is composed of a repeating disaccharide of galactose and *N*-acetylglucosamine with *O*-sulfation at the C-6 position. Both of these sugars are major components of the ES together with sulfate. It has been suggested that a highly sulfated proteoglycan is associated with the epicuticle of the J3 of *Strongyloides ratti* (28,29), and a portion of the negatively charged groups of the cuticle surface of *Caenorhabditis elegans* has been attributed to sulfate residues (23).

Lectin studies of the cuticle surface of *M. javanica* (7) were negative for the monosaccharides detected in the ES in the present study. In a separate study of lectin binding to the cuticular surface of *Meloidogyne* species (27), however, Con A binding was observed, suggesting the presence of glucose or mannose residues on the surface of *M. javanica.* These results indicate that there are likely to be differences between races of *M. javanica,* as described for the other *Meloidogyne* species (27). These findings suggest that the ES originates primarily as a secretory product of the amphids and buccal stylet, both of which were positive for *N*-acetylglucosamine and glucose

or mannose. The absence of lectin binding, however, can result from steric hindrance rather than the absence of a particular monosaccharide residue. Clearly more work is required to elucidate the origin of these ES and the chemistry of their complex carbohydrates and proteins. The ability to collect this material axenically should enable these studies to proceed. Of particular importance will be attempts to raise antibodies to this ES fraction, which should enable a determination of the origin of this complex mixture of molecules.

Literature Cited

1. Aist, S., and R. D. Riggs. 1969. Amino acids from *Heterodera glycines*. Journal of Nematology 1: 254–259.

2. Bartlett, G. R. 1959. Phosphorous assay in column chromatography. Journal of Biological Chemistry 234:466–468.

3. Beeley, J. G. 1985. Glycoprotein and proteoglycan techniques. Laboratory techniques in biochemistry and molecular biology, vol. 16. North-Holland, NY: Elsevier.

4. Bird, A. F. 1975. Plant response to root-knot nematode. Annual Review of Phytopathology 12:69–85.

5. Bird, A. F. 1987. Moulting of parasitic nematodes. International Journal for Parasitology 17:233–239.

6. Bird, A. F., I. Bönig, and A. Bacic. 1988. A role for the "excretory" system in Secernentean nematodes. Journal of Nematology 20:493–496.

7. Bird, A. F., I. Bönig, and A. Bacic. 1989. Factors affecting the adhesion of micro-organisms to the surfaces of plant-parasitic nematodes. Parasitology 98: 155–164.

8. Bird, A. F., W. John, S. Downton, and J. S. Hawker. 1975. Cellulase secretion by second-stage larvae of the root-knot nematode (*Meloidogyne javanica*). Marcellia 38:165–169.

9. Bird, A. F., and B. R. Loveys. 1980. The involvement of ctyokinins in a host–parasite relationship between the tomato (*Lycopersicon esculentum*) and a nematode (*Meloidogyne javanica*). Parasitology 80: 497–505.

10. Bird, A. F., and M. A. McClure. 1976. The tylenchid (Nematoda) egg shell: Structure, composition and permeability. Parasitology 72:19–28.

11. Bird, A. F., and A. C. McKay. 1987. Adhesion of conidia of the fungus *Dilophospora alopecuri* to the cuticle of the nematode *Anguina agrostis*, the vector in annual rye grass toxicity. International Journal for Parasitology 17:1239–1247.

12. Bird, A. F., and D. L. Riddle. 1984. Effect of attachment of *Corynebacterium rathayi* on movement of *Anguina agrostis* larvae. International Journal for Parasitology 14:503–511.

13. Bone, L. W., and K. P. Bottjer. 1985. Cuticular carbohydrates of three nematode species and chemoreception by *Trichostrongylus colubriformis*. Journal of Parasitology 71:235–238.

14. Bradford, M. N. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248–254.

15. Chandrasekaran, E. V., and J. N. BeMiller. 1980. Constituent analysis of glycosaminoglycans. Methods in Carbohydrate Chemistry 8:89–96.

16. Chaplin, M. F. 1982. A rapid and sensitive method for the analysis of carbohydrate components in glycoproteins using gas-liquid chromatography. Analytical Biochemistry 123:336–341.

17. Dische, Z. 1962. Color reactions of hexuronic acid. Methods in Carbohydrate Chemistry 1:497–501.

18. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for the determination of sugars and related substances. Analytical Chemistry 28:350–356.

19. Feizi, T., and R. A. Childs. 1985. Carbohydrate structures of glycoproteins and glycolipids as differentiation antigens, tumor-associated antigens and components of receptor systems. Trends in Biochemical Sciences 10:24–29.

20. Forrest. J. M. S., and W. M. Robertson. 1986. Characterization and localization of saccharide on the head region of four populations of the potato cyst nematode *Globodera rostochiensis* and *G. pallida*. Journal of Nematology 18:23–26.

21. Galambos, J. T. 1967. The reaction of carbazole with carbohydrates. I. Effects of borate and sulfamate on the carbazole color of sugars. Analytical Biochemistry 19:119–132.

22. Gleeson, P. A. 1988. The complex carbohydrates of plants and animals—a comparison. Current Topics in Microbiology and Immunology 139:1–33.

23. Himmelhoch, S., and B. M. Zuckerman. 1983. *Caenorhabditis elegans*: Characters of negatively charged groups on the cuticle and intestine. Experimental Parasitology 55:299–305.

24. Jansson, H-B., A. Jeyaprakash, G. C. Coles, N. Marban-Mendoza, and B. M. Zuckerman. 1986. Fluorescent and ferritin labelling of cuticle surface carbohydrates of *Caenorhabditis elegans* and *Panagrellus redivivus*. Journal of Nematology 18:570–574.

25. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680–685.

26. McClure, M. A., and B. M. Zuckerman. 1982. Localization of cuticular binding sites of Concanavalin A on *Caenorhabditis elegans* and *Meloidogyne incognita*. Journal of Nematology 14:39–44.

27. McClure, M. A., and B. A. Stynes. 1988. Lectin binding studies on the amphidial exudates of *Meloidogyne*. Journal of Nematology 20:321–326.

28. Murrell, K. D., and C. Graham. 1982. Solubilization studies on the epicuticular antigens of *Strongyloides ratti*. Veterinary Parasitology 10:191–203.

29. Murrell, K. D., C. E. Graham, and M. McGreevy. 1983. *Strongyloides ratti* and *Trichinella spiralis*: Net charge of epicuticle. Experimental Parasitology 55:331–339.

30. Myers, R. F., and L. R. Krusberg. 1965. Or-

ganic substances discharged by plant-parasitic nematodes. Phytopathology 55:429–437.

31. Nagasawa, K., and Y. Inoue. 1980. De-*N*-sulphation. Methods in Carbohydrate Chemistry 8:291–294.

32. Nelson, F. K., and D. L. Riddle. 1984. Functional study of the *Caenorhabditis elegans* secretory–excretory system using laser microsurgery. Journal of Experimental Zoology 231:45–56.

33. Nordbring-Hertz, B. 1983. Mycelial development and lectin–carbohydrate interaction in nematode-trapping fungi. Pp. 419–432 *in* D. H. Jennings and A. D. M. Rayner, eds. Ecology and physiology of the fungal mycelium. London: Cambridge University Press.

34. Olden, K., B. A. Bernard, M. J. Humphries, T-K. Yeo, K-T. Yeo, S. L. White, S. A. Newton, H. C. Bauer, and J. B. Parent. 1985. Function of glycoprotein glycans. Trends in Biochemical Sciences 10:78–82.

35. Premachandran, D., N. Von Mende, R. S. Hussey, and M. A. McClure. 1988. A method for staining nematode secretions and structures. Journal of Nematology 20:70–78.

36. Spiegel, Y., E. Cohn, and S. Spiegel. 1982. Characterization of sialyl and galactosyl residues on the body wall of different plant parasitic nematodes. Journal of Nematology 14:33–39.

37. Stirling, G. R., A. F. Bird, and A. B. Cakurs. 1986. Attachment of *Pasteuria penetrans* spores to the cuticles of root-knot nematodes. Revue de Nématologie 9:251–260.

38. Veech, J. A., J. L. Starr, and R. M. Nordgren. 1987. Production and partial characterization of stylet exudate from adult females of *Meloidogyne incognita.* Journal of Nematology 19:463–468.

39. Wang, E. L. H., and G. B. Bergeson. 1978. Amino acids and carbohydrates secreted by *Meloidogyne incognita.* Journal of Nematology 10:367–368.

40. Wright, K. A. 1987. The nematode's cuticle—its surface and the epidermis: Function, homology, analogy—a current consensus. Journal of Parasitology 73:1077–1083.

41. Zuckerman, B. M., and H-B. Jansson. 1984. Nematode chemotaxis and possible mechanisms of host/prey recognition. Annual Review of Phytopathology 22:95–113.

42. Zuckerman, B. M., I. Kahane, and S. Himmelhoch. 1979. *Caenorhabditis briggsae* and *C. elegans*: Partial characterization of cuticle surface carbohydrates. Experimental Parasitology 47:419–424.