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Glycoprotein Characterization of the Gelatinous Matrix in the Root-knot Nematode *Meloidogyne javanica*[1]

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Abstract: Proteinaceous components of freshly formed gelatinous matrix (GM) of the root-knot nematode *Meloidogyne javanica* were analyzed. Under reducing conditions, the prominent protein fragments had molecular weights of 26 to 66 kDa and 150 to >200 kDa, and most were glycosylated. Most of the fragments were digested by proteinase K, and fewer by trypsin. The lectins soybean agglutinin (SBA), *Ulex europaeus* agglutinin, and wheat germ agglutinin labeled the higher molecular weight bands (i.e., >200 kDa). SBA labeled additional protein fractions between 26 and 66 kDa. Although *Bandeiraea simplicifolia* lectin and Concanavalin A did not label bands on the Western blot, they did label the GM in the dot blot technique. Analysis of amino acids and amino sugars in the GM revealed an unusually high amount of ammonia and galactosamine moieties.

Key words: gelatinous matrix, glycoprotein, lectin, *Meloidogyne javanica*, nematode, protein.

Females of several sedentary phytoparasitic nematodes deposit their eggs within a gelatinous matrix (GM). In the root-knot nematodes, *Meloidogyne* spp., GM is synthesized in a voluminous quantity by the female anal glands and secreted through the anus immediately before and during egg laying; eggs are deposited into the GM to form the egg mass (8). Information about the chemical composition of the matrix is very limited; the *Meloidogyne* GM appeared to be morphologically homogeneous and to be composed largely of a tanned protein containing carbohydrate (6,11) but no lipids (1,2).

Recently, microorganisms and human red blood cells (HRBC) were shown to agglutinate in the presence of the GM; this agglutination could partially explain the GM ability to protect the eggs from surrounding microorganisms in soil (10). Therefore, we chose to characterize further the compounds within the GM to deepen our understanding of this agglutination phenomenon.

MATERIALS AND METHODS

Gelatinous matrix: Meloidogyne javanica was monoxenically cultured on excised roots of tomato (*Lycopersicon esculentum*) cv. Hosen Eilon (5). Twenty-five days after roots were inoculated with nematode eggs, freshly formed GM was drawn with a sterile Pasteur pipet from 200 egg masses and dissolved in 1 ml double distilled water (or in 0.1 M Tris, pH 6.8, for polyacrylamide gel electrophoresis [PAGE]). The solution was then centrifuged at 1,000*g* for 1 minute, and the supernatant fraction was separated from the eggs by decantation. The GM at this particular age was still clear and lacked the typical viscoidal nature of the mature GM; only a few eggs were deposited by this stage, and after centrifugation the GM solution did not contain eggs. Total protein concentration of the GM solution was determined with the Bradford (3) method.

Amino acid and amino sugar analysis: Nematode GM solution (650 µg/ml protein) was hydrolyzed in 6 N HCl for 22 hours at 110 C under nitrogen atmosphere (9). The amino acid compositions were determined with a Biotronic LC 5000 amino acid analyzer with a post-run derivatization with ninhydrin. For cystine determination, fractions were preoxidized with performic acid (9). The analyses were repeated two times with GM from different harvests.

Gel electrophoresis and Western blots: Analytical PAGE in 7 and 12% gels containing 0.1% sodium dodecyl sulfate (SDS) was performed in a Mini-Protean II apparatus (Bio-Rad Laboratories, Richmond, CA),

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using 1.5-mm-thick vertical slab gels essentially as described by Laemmli (7), with 40 µl GM solution loaded per well (= 26 µg protein). Gels were silver stained (12). Molecular-weight markers (Bio-Rad) were used in silver-stained gels, and prestained markers (Sigma Chemical Co., St. Louis, MO) were used in each gel to be electroblotted. For Western blots, proteins were transferred onto nitrocellulose sheets using a Gelman Sciences BioTranse (Ann Arbor, MI) midi apparatus.

Labeling of glycoconjugates: A highly sensitive periodic acid–silver stain was used for PAGE-separated proteins, for detection of 1,2-diol groups of glycoproteins and polysaccharides (4).

Specific carbohydrates were labeled on the electroblotted nitrocellulose with peroxidase-conjugated lectins. The lectins used were *Bandeiraea simplicifolia* (BS-I), Concanavalin A (Con A) (*Canavalia ensiformis* agglutinin), soybean (*Glycine max*) agglutinin (SBA), *Ulex europaeus* agglutinin (UEA-I), and wheat germ (*Triticum vulgaris*) agglutinin (WGA). Blocking was performed with 0.15 M Tris-buffered saline, pH 7.4 (TBS), containing 1% bovine serum albumin (BSA), and 0.05% Tween 20 for 1 hour at room temperature (RT). The membrane was then washed three times for 10 minutes with TBS containing 0.1% BSA and 0.05% Tween 20. Blocked membranes were incubated with peroxidase-conjugated lectins (2 µg/ml TBS containing 0.1% BSA) for 1 hour at RT and then washed three times with TBS containing 0.1% BSA. To visualize the labeling, 4-chloro-1-naphthol was used as a substrate. Lectins that had been preincubated (for 30 minutes) with 0.1 M of the appropriate inhibitory saccharide (namely, D-galactose for BS-I, α-methyl mannopyrannoside or glucose for Con A, N-acetylgalactosamine for SBA, fucose for UEA, and N-acetylglucosamine for WGA) and a GM solution unexposed to lectin were used simultaneously as controls.

Dot blot tests were performed by preparing dilutions to one, two, and four times original volume (i.e., ×1, ×2, ×4) of aqueous GM solution containing 650 µg/ml protein and pipetting 10 µl of each concentration onto the nitrocellulose in an ordered array. The drops were air-dried for 15 minutes, blocked, incubated with the peroxidase-conjugated lectins, and visualized as described in the glycoconjugate labeling procedure described above. Lectins that had been pre incubated for 30 minutes with 0.1 M of the appropriate inhibitory saccharide, and GM unexposed to lectins, were used simultaneously as controls.

Enzyme digestions: Proteins (650 µg) in the GM, dissolved in 1 ml of 0.1 M Tris (pH 8.0) containing 1 mM $CaCl_2$, were digested for 3 hours at 37 C by incubation with 20 µg/ml proteinase K (from *Tritirachium album,* Boehringer Mannheim, Mannheim, Germany) or 20 µg/ml trypsin (Sigma).

Results

Amino acid analysis: The most notable finding during analysis of the acid hydrolyzate of *M. javanica* GM solution was the unusually high amount of ammonia and galactosamine moieties (16.5% and 45.9%, respectively, Table 1). The ammonia was probably released from asparagine and glutamine during acid hydrolysis.

Electrophoresis: The SDS-PAGE separation of the GM solution was repeated several times at two gel strengths, and a typical fractionation is presented in Figure 1. The most prominent proteins had molec-

Table 1. Amino acids and related compounds detected in acid hydrolyzate of an aqueous solution of *Meloidogyne javanica* gelatinous matrix (650 µg/ml protein).

Compound	% in sample	Compound	% in sample
Alanine	1.02	Phenylalanine	0.76
Arginine	0.57	Proline	3.28
Aspartic acid	8.87	Serine	1.40
Glutamic acid	8.93	Threonine	3.40
Glycine	1.76	Tyrosine	0.42
Histidine	0.48	Valine	0.91
Isoleucine	0.71		
Leucine	0.76	Ammonia	16.54
Lysine	1.73	Galactosamine	45.95
Methionine	0.05	Glucosamine	2.45

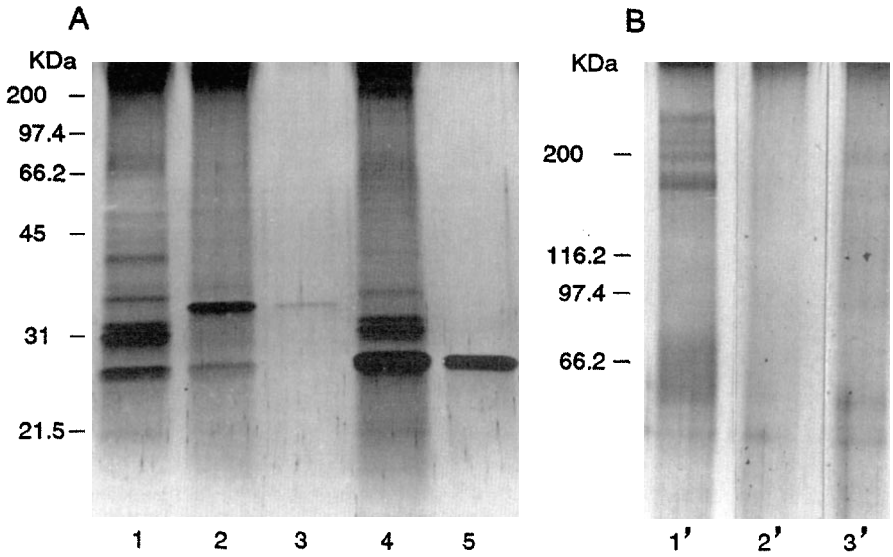

FIG. 1. *Meloidogyne javanica* gelatinous matrix (GM) treated with proteolytic enzymes, fractionated on 12 (A) and 7% (B) polyacrylamide gels, and stained with silver. Lanes 1 and 1', untreated GM; lanes 2 and 2', proteinase K-treated GM; lanes 4 and 3', trypsin-treated GM; lanes 3 and 5, proteinase K and trypsin, respectively.

ular weights (MW) of 26, 31, 32, 35, 40, 48, 66, 150, 180, 200, and >200 kDa, and most were glycosylated (Fig. 2). The lectins SBA and UEA-I (Fig. 2) and WGA (very faint band, not shown) labeled the higher m.w. bands, namely, >200 kDa, although the SBA labeling of the >200 kDa band was very faint. SBA also labeled protein fractions between 26 and 66 kDa (Fig. 2), whereas BS-I and Con A did not label the Western blot. Labelings by SBA, UEA-I, and WGA were completely inhibited by the appropriate haptens.

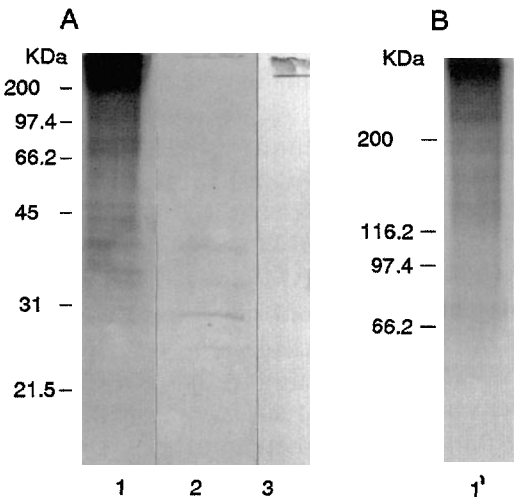

FIG. 2. *Meloidogyne javanica* gelatinous matrix fractionated on a 12 (A) and 7% (B) SDS-PAGE. Lanes 1 and 1', gels stained for carbohydrate residues by periodic acid-silver; lanes 2–3, Western blots of gel (A) labeled with peroxidase conjugates of soybean agglutinin (lane 2) and *Ulex europaeus* agglutinin (lane 3).

The dot blot method produced different intensities of labelings with BS-I, Con A, SBA, UEA-I, and WGA; labelings were inhibited by the appropriate haptens (Fig. 3, Table 2). GM unexposed to lectins did not exhibit any labeling.

Proteolytic digestion: Most of the bands were digested by proteinase K, although several minor bands remained intact (Fig. 1). Trypsin digested most of the higher MW bands (>66 kDa) but barely digested the bands <66 kDa (Fig. 1).

DISCUSSION

In this work we analyzed the electrophoretic patterns of proteins or glycoproteins of freshly formed GM collected from *M. javanica*-infected roots cultured monoxenically. Previous analyses of the chem-

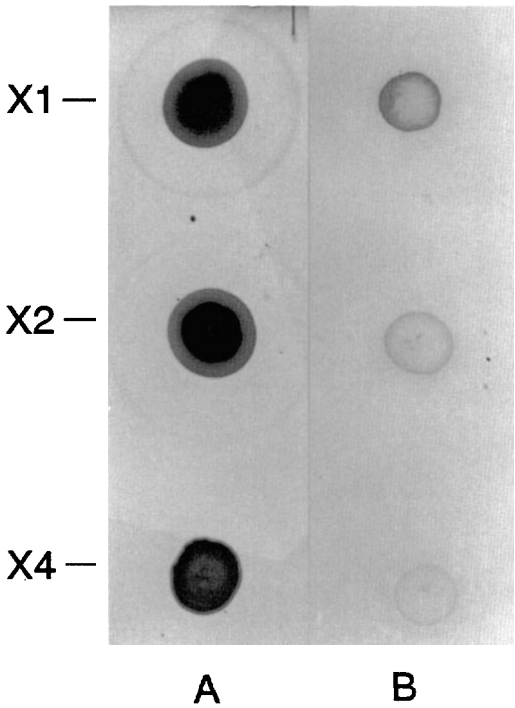

FIG. 3. Dot blot test for the presence of *Ulex europaeus* agglutinin (UEA-I)-binding carbohydrate in the gelatinous matrix (GM) of *Meloidogyne javanica*. The test was performed by preparing double dilutions (×1, ×2, ×4) of aqueous GM solution containing 650 µg/ml protein and pipetting 10 µl of each concentration onto the nitrocellulose in an ordered array. The drops were air-dried for 15 minutes, bloked, incubated with the peroxidase-conjugated lectins, and visualized with 4-chloro-1-naphtol. A) UEA-I binding to the GM. B) Control blot: the lectin was preincubated for 30 minutes with 0.1 M of L-fucose, the appropriate inhibitory hapten.

ical composition of the *Meloidogyne* GM were performed on mature GM containing eggs and dissected from infected plants grown under nonsterile conditions (6,11).

The presence of galactose/N-acetylgalactosamine and glucose/N-acetylglucosamine moieties in *M. javanica* GM had already been detected (6,11), using the appropriate lectins. Although a high intensity of WGA labeling on the GM surface has been reported (6), our present work revealed only weak labeling with this lectin. Our present labeling results agree with the low amount (2.45%) of glucosamine residues found in the amino acid analysis. This difference between earlier research

TABLE 2. Lectin binding in dot blot tests performed with a solution of *Meloidogyne javanica* gelatinous matrix.

Lectin†	Inhibitory hapten	Intensity of labeling: Without hapten	Intensity of labeling: With hapten
BS-I	D-Galactose	+	—
Con A	Glucose or mannose	+	—
SBA	N-Acetylgalactosamine	+ + + +	+
UEA-I	L-Fucose	+ + + +	+
WGA	N-Acetylglucosamine	+ +	—

Dot blot tests were performed by preparing dilutions (×2 and ×4 = dilutions to two and four times original volume) of aqueous GM solution containing 650 µg/ml protein and pipetting 10 µl of each concentration onto the nitrocellulose in an ordered array. The drops were air-dried for 15 minutes, blocked, incubated with the peroxidase-conjugated lectins, and visualized with 4-chloro-1-naphtol. GM unexposed to lectins did not reveal any labeling.

† BS-I = *Bandeiraea simplicifolia* lectin; Con A = concanavalin A; SBA = soybean agglutinin; UEA-I = *Ulex europaeus* agglutinin; WGA = wheat germ (*Triticum vulgaris*) agglutinin.

+, + +, + + + + = increasing intensities of labeling.
— = no labeling.

(6) and the present report may result from the age of the GM or different methodologies. Mature GM may contain more glucosamine residues than immature GM, or these residues may be more exposed on the surface of mature GM.

The electrophoretic patterns showed that most of the proteins of *M. javanica* GM are digested by proteolytic enzymes. Because most of the bands stained for carbohydrate moieties in Figure 2 are protease digestable (Fig. 1), we assume that the GM is composed of many glycosylated proteins. The amino sugar profile, with a high amount of galactosamine residues (ca 46%), is consistent with the high intensity of SBA labeling. Moreover, lipid analysis has shown that lipid does not exist in the GM (2).

Therefore, we suggest that the GM protein pattern comprises low- and high-MW glycoproteins, which might play a role in the previously described (10) agglutination phenomenon of HRBC and microorganisms.

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