Journal of Nematology 29(3):276–281. 1997.

Unique Immunogenic Proteins In *Heterodera glycines* Eggshells[1]

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Abstract: Polyclonal antibodies were raised against *Heterodera glycines* eggshells to determine the feasibility of developing an immunoassay for *H. glycines* eggs. An indirect enzyme-linked immunosorbent assay (ELISA) was developed from antisera collected 10 weeks after the initial injection. From serial dilutions of sonicated eggshells or whole eggs, a sensitivity of detection to 5 ng/ml sonicated eggshells or 1 egg of *H. glycines* was determined. The method of eggshell preparation had no effect on the antibodies produced; however, the antibodies cross-reacted with sonicated J2 of *H. glycines* and eggs of *Meloidogyne incognita* and *H. schachtii*. Most of the proteins in both life stages of *H. glycines* and eggs of *M. incognita* and *H. schachtii* had similar migration properties when separated on SDS-PAGE gels and stained with Coomassie blue. Western blot analysis, with antisera adsorbed with homogenized J2 of *H. glycines*, showed proteins that were specifically localized to eggshells of *H. glycines*. Monoclonal antibodies might provide a useful immunoassay where polyclonal antibodies lack sufficient specificity.

Key words: adsorbed antisera, ELISA, hatching, *Heterodera glycines*, immunoassay, nematode.

Immunoassays are routinely used to detect and quantify plant and animal pathogens because they reduce the amount of time needed for identification while increasing the reliability of diagnoses. For example, polyclonal antibodies raised against homogenates of *Bursaphelenchus xylophilus* could detect approximately 25 nematodes on the surface of debarked wood sections (Lawler and Harmey, 1993). However, the antibody was unable to distinguish between *B. xylophilus* and *B. mucronatus*. Previous studies have demonstrated that immunoassays for various life stages of nematodes can differentiate species (Fox and Atkinson, 1985; Scott and Riggs, 1971; Webster and Hooper, 1968), even though examples of cross-reactivity exist. Antibodies raised against extracts from cysts of *Heterodera* species were used to separate, by agar-gel double diffusion, the two morphology-based groups of *Heterodera* (Webster and Hooper, 1968). Immunoelectrophoresis revealed different patterns for *H. glycines* and *H. betulae* (Scott and Riggs, 1971) that distinguished between the two species. Additionally, crossed immunoelectrophoresis was able to separate *Globodera rostochiensis* and *G. pallida* (Fox and Atkinson, 1985).

Although polyclonal antibodies raised against cyst extracts could be used in agar-gel double diffusion or immunoelectrophoresis to separate species, it has not been determined whether they can be used to quantify nematodes. Schots et al. (1992) used monoclonal antibodies in an enzyme-linked immunosorbent assay (ELISA) to detect and quantify eggs of *G. pallida* and *G. rostochiensis*. With three monoclonal antibodies and the binding constants for each antibody, 1 to 100 eggs could be detected and identified. Because polyclonal antibodies to homogenates of cysts of *Heterodera* species could be used to differentiate species, our objective was to test the feasibility of using polyclonal antibodies to detect and quantify eggs of *H. glycines*.

MATERIALS AND METHODS

Antigen preparation: Eggs of *H. glycines* were collected as described by Yen et al. (1995). The eggs were surface-sterilized in 0.1% chlorhexidine diacetate for 10 minutes, thoroughly rinsed with sterilized water, and placed on sterilized 30-µm-pore nylon mesh sieves suspended over a 1:10 dilution of 15 mM $ZnSO_4$ to encourage hatching. After 2 days the solution was changed to 30 mM

Received for publication 30 December 1996.

[1] Contribution from the Missouri Agricultural Experiment Station, Journal Series No. 12,588. This research was partially supported by the Misouri Soybean Merchandising Council, the Missouri Agricultural Experiment Station, and USDA Special Grant 90-34113-5000.

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The authors thank Robert D. Heinz, Office of Lab Animal Medicine, University of Missouri-Columbia; Yehsiung Sha; and H. B. Krishnan for assistance.

$ZnSO_4$. The solution was changed every 2 days for 22 days and second-stage juveniles (J2) were removed. Eggshells remaining on the sieves were collected and centrifuged at 1,100*g* in a 40% to 100% sucrose density-step gradient to remove debris, J2, and unhatched eggs. The eggshells were washed with deionized water and homogenized in a glass tissue homogenizer or disrupted by sonication with a Brinkmann homogenizer (Brinkmann Instrumentation, Switzerland).

Production of antibodies: New Zealand White rabbits were injected with homogenized or sonicated eggshells in Freund's complete adjuvant at a concentration of 1 mg eggshells/ml adjuvant. Six and 13 weeks after the initial injection, rabbits were injected with the antigen in Freund's incomplete adjuvant. Blood was collected at 10-week intervals, with final collection 22 weeks after initial injection. To remove antibodies against J2 antigens that might have contaminated the preparations, 1 ml of homogenized eggshell antiserum was incubated with 150 µg of sonicated J2 of *H. glycines* with agitation at 30 °C for 30 minutes. The precipitate was removed by centrifugation at 2,000g for 10 minutes. The supernatant was collected, and the adsorption was repeated twice (Hussey, 1972).

Indirect enzyme-linked immunosorbent assay (ELISA): Indirect ELISA was used to determine antibody titer, sensitivity to sonicated and whole eggs, and species-specificity of both the original antisera and the adsorbed sera. All assays were performed as described by Clark and Adams (1977), except where noted. The secondary antibody was goat anti-rabbit IgG-horseradish peroxidase (Promega, Madison, WI). Substrates used were O-phenylenediamine (dihydrochloride) (Sigma, St. Louis, MO) in 60.8 mM Na_2HPO_4, 26.6 mM citric acid, 0.012% H_2O_2 (pH 5.0), or 2,2′-azino-bis-(3-benzthiazoline-6-sulfonic acid) (ABTS, Southern Biotechnology Associates, Birmingham, AL) in 54.7 mM citric acid (pH 4.0) or 3.3% H_2O_2 (where noted). Absorbance was read at 450 nm or 405 nm (ABTS) at 10, 20, and 30 minutes on a microplate reader (Titertek Multiskan Plus, Flow Laboratories, McLean, VA).

To determine antibody titer, a 1-µg/ml suspension of sonicated eggshells was added to each well of a microtiter plate. Two-fold serial dilutions of each antibody, adsorbed sera, or pre-immune serum were subsequently added to duplicate wells. Antibody sensitivity was determined by screening various dilutions of sonicated eggshells in duplicate, with a 1:16,000 dilution of the antisera or a 1:4,000 dilution of adsorbed sera. Additionally, two-fold serial dilutions of eggs (either sonicated or whole and stored at −20 °C) were screened with a 1:16,000 dilution of antibody. Where dilutions of whole eggs (stored at 4 °C) were screened with a 1:16,000 dilution of the antisera or the 1:4000 dilution of the adsorbed antiserum, ABTS substrate was used.

Species and life-stage specificity of the antisera were determined in two-fold dilutions of 70 ng/ml of sonicated antigen added to duplicate wells containing *H. glycines* J2 or eggs, *M. incognita* eggs, or *H. schachtii* eggs. The antigens were incubated with a 1:16,000 dilution of antisera or a 1:4,000 dilution of adsorbed antisera, and ABTS substrate was used.

SDS-PAGE and Western blot: Coomassie blue-stained SDS-polyacrylamide gels (Laemmli, 1970) and Western blot analysis (Towbin et al., 1979) were used to characterize antigenic differences and antibody specificity. Polyacrylamide slab gels were cast with a 4% stacking phase and a 13.5% separating phase. Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Aliquots containing 25 µg of protein derived from sonicated J2 of *H. glycines,* eggshells of *H. glycines, M. incognita* eggs, or *H. schachtii* eggs were loaded into the gel. Electrophoresis was at 25 mA constant current, until samples entered the separating gel. At that point current was increased to 35 mA until the loading dye reached the end of the gel. Two gels were electrophoresed in parallel. One gel was stained with Coomassie blue (methanol, glacial acetic acid, water [5:1:5], 0.5% (w/v) Coomassie brilliant blue R250)

overnight at room temperature with agitation. The gel was destained in 10% acetic acid and 10% methanol. Proteins on the second gel were transferred to a nitrocellulose membrane with a Bio-Rad TransBlot (Bio-Rad Laboratories, Hercules, CA). After transfer, the membrane was cut in two and probed as described by Towbin et al. (1979) with a 1:250 dilution of the adsorbed antisera or the non-adsorbed sera. The antibodies were detected with goat-anti-rabbit IgG-horseradish peroxidase at a 1:3,000 dilution (Bio-Rad Laboratories, Richmond, CA) and were visualized with 60 mg 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, CA) in 20 ml methanol, 60 µl 30% H_2O_2, and 100 ml TBS.

RESULTS

ELISA: The homogenized and sonicated eggshell preparations were equally effective immunogens; there was little difference between the two preparations. Absorbance at A_{450} nm for both antisera became saturated at dilutions between 1:1,000 and 1:8,000. Absorbance sharply decreased for dilutions of the unadsorbed antisera greater than 1:16,000, but was higher than the reaction of the pre-immune sera for both sera even at a 1:512,000 dilution. Therefore, 1:16,000 dilutions of unadsorbed antisera were used in subsequent experiments. The A_{405} curve of the adsorbed antisera was saturated at a 1:2,000 dilution and gradually decreased to a dilution of 1:128,000. A 1:4,000 dilution of the adsorbed antisera was used in the species-specificity assay, and a 1:16,000 dilution was used with whole eggs.

Both antisera detected as little as 5 ng/ml of sonicated eggshells (data not shown). Neither antiserum, however, could be used to distinguish concentrations of sonicated eggshells greater than 50 ng/ml because the reactions became saturated at that point. The adsorbed antisera did not reveal a greater range of sensitivity.

As few as 8 eggs/ml, or 1 egg picked microscopically (A_{405} nm = 0.2) that had been stored at −20°C, could be detected. The reactions of both antisera to eggs stored at −20 °C were saturated at concentrations greater than 62 eggs/ml and were indistinguishable. However, the range distinguished by both the adsorbed antisera and unadsorbed antisera to whole eggs stored at 4 °C was larger (between 50 eggs/ml and 400 eggs/ml) than the range for eggs stored at −20 °C.

Both unadsorbed antibodies reacted with J2 of *H. glycines,* as well as with eggs of *M. incognita* and *H. schachtii* (Fig. 1A), although the three species could be distinguished at protein concentrations of 70 ng/ml. A substantial difference was not observed between the eggs and J2 of *H. glycines* at the lower concentrations. However, the A_{405} at 70 ng/ml for the J2 was 0.90, and the A_{405} for eggs of *H. glycines* was 0.55. Although the differences in A_{405} decreased as the antigen concentration decreased, the A_{405} for eggs of *M. incognita* and the *Heterodera* spp. differed at 8.75 ng/ml. At concentrations lower than 8.75 ng/ml, no differences were detected among species or life stages.

The adsorbed antisera also reacted with J2 and eggs of *H. glycines,* and eggs of *M. incognita* and *H. schachtii* (Fig. 1B). There were, however, distinct differences between the eggs of *M. incognita* and *H. glycines* at all concentrations at A_{405}. There were no consistent differences between the eggs and J2 of *H. glycines* at concentrations greater than 62 ng/ml at A_{405}. The A_{405} for eggs of *H. schachtii* was distinctly different from that of eggs of *M. incognita.*

SDS-PAGE and Western blot: When an SDS-PAGE gel was stained with Coomassie blue, a number of protein bands were observed that were common to J2 and eggshells of *H. glycines,* and to eggs of *H. schachtii* and *M. incognita.* Western blot analysis indicated that the antibodies raised against eggshells of *H. glycines* were likely polyclonal (Fig. 2). The unadsorbed antisera bound to proteins in all four preparations (data not shown). In contrast, the adsorbed antisera revealed six proteins with specificity to the eggshells of *H. glycines* and *H. schachtii,* and another four with specificity for *H. glycines* eggshells. Proteins of 36, 31, 30, 28, 19, and 9 kda were unique to the eggshell preparations from *H.*

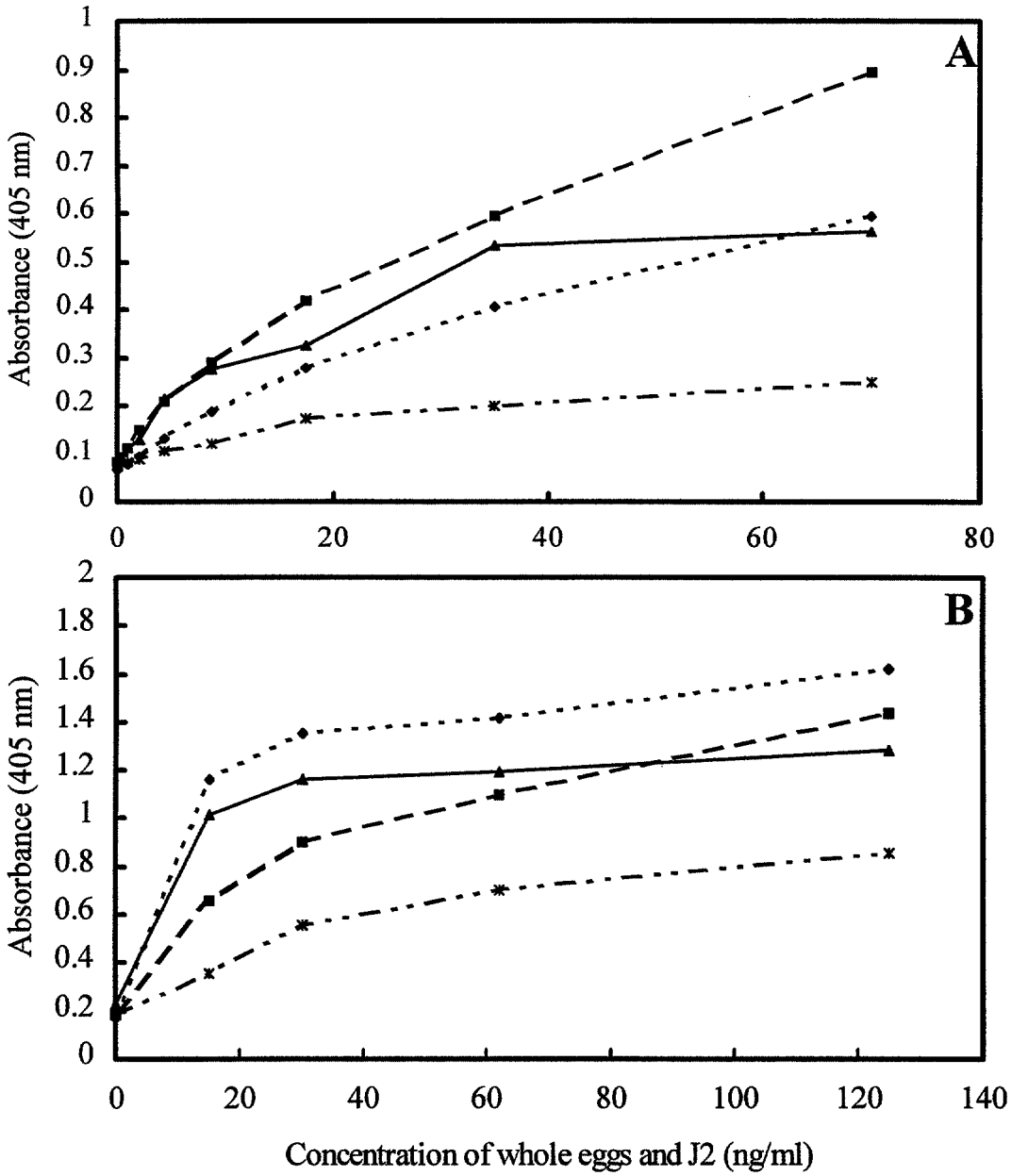

FIG. 1. Species and life-stage specificity of unadsorbed antiserum or adsorbed with extracts of J2 *Heterodera glycines*. Polyclonal antibodies were raised against a homogenized preparation of eggshells of *Heterodera glycines*. Specificity was determined by an indirect enzyme-linked immunosorbent assay. Absorbance was read at 405 nm after 20 minutes' incubation with the substrate. A) A 1:16,000 dilution of homogenized antiserum used to determine species and life-stage specificity of two-fold serial dilutions of sonicated nematode life stages. B) A 1:4,000 dilution of the adsorbed antiserum used to determine species and life-stage specificity of two-fold serial dilutions of sonicated nematode life stages. Legend: *H. glycines* J2 (—■—), *H. glycines* eggs (—◆—), *M. incognita* eggs (—*—), *H. schachtii* eggs (—▲—).

glycines and *H. schachtii* (Fig. 2, lanes 2,3); proteins of 65, 43, 28.8, and 11 kda were specific to the eggshells of *H. glycines* (Fig. 2, lane 3).

DISCUSSION

It is clear from ELISA analysis that eggshells of *H. glycines* can be used as an effec-

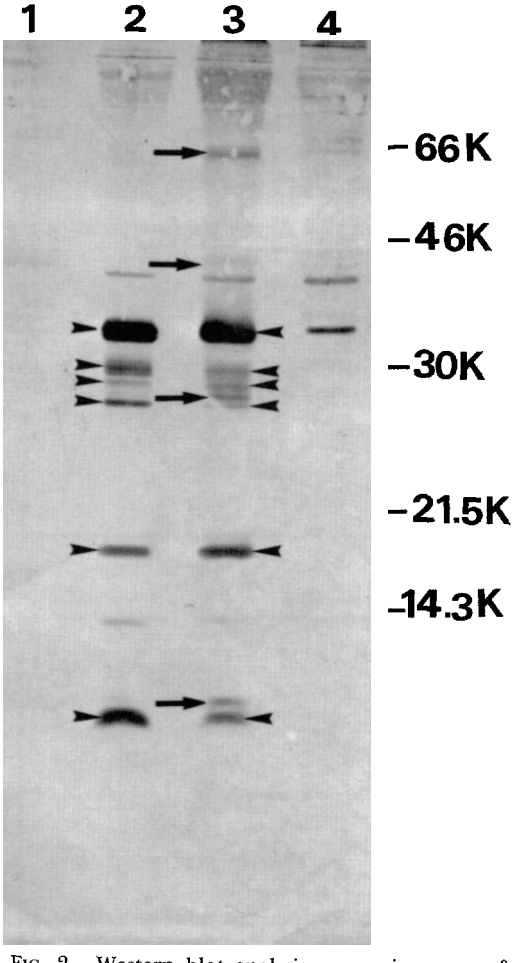

FIG. 2. Western blot analysis comparing eggs of *Meloidogyne incognita* (lane 1), *H. schachtii* (lane 2), *H. glycines* eggs (lane 3), and second-stage juveniles (J2) of *Heterodera glycines* (lane 4). The antiserum was raised against a homogenized preparation of eggshell of *H. glycines* and adsorbed with J2 of *H. glycines*. The 36-, 31-, 30-, 28-, 19-, and 9-kda proteins (▶) are unique to the eggshell preparations of *H. glycines* and *H. schachtii*. The 65-, 43-, 28.8-, and 11-kda proteins (➜) are present in preparations of eggshells of *H. glycines* and are absent in the other preparations.

tive immunogen. However, the range of concentrations of antisera that can be used for quantification of eggs extracted from a soil sample is too narrow to be a useful diagnostic assay. The greater range in sensitivity seen when eggs were stored at 4 °C compared to frozen eggs indicates that some of the immunogens were diffusible and could be solubilized by freeze-thaw cycles. Because the eggshells injected into the rabbits were sonicated or homogenized, the diffusible immunogens would be more accessible to elicit an antigenic response than if eggs were intact. Although polyclonal antibodies have been used to distinguish species of *Heterodera* (Scott and Riggs, 1971) and *Globodera* (Fox and Atkinson, 1985), quantification of eggs in the sample must be done separately. Also, the polyclonal antibodies to the eggshells of *H. glycines* lacked life-stage specificity. The greater reaction of the antisera to J2 than to eggs of *H. glycines* would lead to an inaccurate estimate of the population density if J2 were present in the soil sample. Although the antisera adsorbed against J2 proteins differentiated a greater range of concentrations, the reaction to the J2 was not substantially different from that to eggs of *H. glycines*, consequently, even this partially purified antiserum would lead to inaccurate estimates of the population density, as well. The lack of species-specificity of both the antisera and partially purified antisera does not improve upon current methods of species identification.

It may be possible to develop an assay using monoclonal antibodies, similar to that of Schots et al. (1992) for eggs of *G. rostochiensis* and *G. pallida*. Eggs of *H. glycines* show structural similarities to eggs of *G. rostochiensis*, as well as a hatching response to zinc similar to the *G. rostochiensis* response to calcium (Perry and Trett, 1986). These similarities indicate that unique proteins present in the eggs could have a role in hatching and host-specific responses, and that a monoclonal antibody specific to eggshells of *H. glycines* might be identified.

Western blot analysis revealed proteins that are specific to *H. glycines* eggshells. It should be possible to obtain monoclonal antibodies to these proteins. Evidence obtained from *G. pallida* (Backett et al. 1993) revealed that a monoclonal antibody that is life-stage and species-specific could be raised to the unique proteins. Monoclonal antibodies have been shown to be more useful for diagnostic assays than polyclonal antibodies (Halk and De Boer, 1985). Monoclonal antibodies also could be used to localize the unique proteins in the eggs of *H. gly-*

cines, and might provide information on the mechanism of hatching.

Literature Cited

Backett, K. D., H. J. Atkinson, and J. M. S. Forrest. 1993. Stage-specific monoclonal antibodies to the potato cyst nematode *Globodera pallida* (Stone) Behrens. Journal of Nematology 25:395–400.

Clark, M. F., and A. N. Adams. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. Journal of General Virology 34:475–483.

Fox, P. C., and H. J. Atkinson. 1985. Immunochemical studies on pathotypes of the potato cyst nematodes, *Globodera rostochiensis* and *G. pallida.* Parasitology 90: 471–483.

Halk, E. L., and S. H. De Boer. 1985. Monoclonal antibodies in plant disease research. Annual Review of Phytopathology 23:321–350.

Hussey, R. S. 1972. Serological relationship of *Meloidogyne incognita* and *M. arenaria.* Journal of Nematology 4:101–104.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lawler, C., and M. A. Harmey. 1993. Immunological detection of nematode antigens on the surface of a wood section. Fundamental and Applied Nematology 16:521–523.

Perry, R. N., and M. W. Trett. 1986. Ultrastructure of the eggshell of *Heterodera schachtii* and *H. glycines* (Nematoda: Tylenchida). Revue de Nématologie 9: 399–403.

Schots, A., F. J. Gommers, and E. Egberts. 1992. Quantitative ELISA for the detection of potato cyst nematodes in soil samples. Fundamental and Applied Nematology 15:55–61.

Scott, H. A., and R. D. Riggs. 1971. Immunoelectrophoretic comparisons of three plant-parasitic nematodes. Phytopathology 61:751–752.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proceedings of the National Academy of Sciences of the U.S.A. 76:4350–4354.

Webster, J. M., and D. J. Hooper. 1968. Serological and morphological studies on the inter- and intraspecific differences of the plant-parasitic nematodes *Heterodera* and *Ditylenchus.* Parasitology 58:879–891.

Yen, J. H., T. L. Niblack, and W. J. Weibold. 1995. Dormancy of *Heterodera glycines* in Missouri. Journal of Nematology 27:153–163.