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Detection and Partial Characterization of Egg Polypeptides from *Heterodera glycines*

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Abstract: The presence of two major egg polypeptides was demonstrated in the plant-parasitic nematode *Heterodera glycines*. The polypeptides were present in equal amounts in, and were most abundant in, eggs from yellow females. They were also present in brown females but were not detected in second-stage juveniles (J2). The two major egg polypeptides, MEP-I and MEP-II, accounted for more than 50% of the total protein in egg extracts evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. During development of females from the yellow stage to the brown stage, the levels of MEP-I and MEP-II declined at twice the rate as total protein. MEP-I and MEP-II had estimated molecular masses of 190 kD and 180 kD, respectively, similar to those reported for female-specific proteins, vitellins, from free-living nematodes.

Key words: Caenorhabditis elegans, Dolichorhabditis sp., egg, electrophoresis, female development, *Heterodera glycines,* major egg polypeptide, nematode, protein, reproductive physiology, soybean cyst nematode, vitellin, vitellogenin.

Developing oocytes in most oviparous animals accumulate large amounts of protein that are deposited as yolk components and serve as nutritional stores for embryogenesis (Kunkel and Nordin, 1985; Wahli et al., 1981; Wallace, 1985). These proteins are synthesized extraovarially in organs such as the vertebrate liver (Wallace, 1985), insect fat body (Kunkel and Nordin, 1985), and nematode intestine (Kimble and Sharrock, 1983), usually as large precursor polypeptides, vitellogenins, which are transported to developing oocytes. Oocyte uptake is selectively accomplished through receptor-mediated endocytosis (Byrne et al., 1989; Sappington and Raikhel, 1995). Chemical modifications (e.g., glycosylation, lipidation, proteolysis) may be applied to the precursor vitellogenins prior to or during endocytosis, depending upon phylum (Chen et al., 1997). Some vitellogenins are sequestered by the oocyte without modification. Once deposited within the oocyte, the mature polypeptides, modified or not, are called vitellins. These female-specific proteins are essential elements of reproductive physiology. While there is no typical vitellin, in the lower invertebrates vitellins have been most thoroughly studied in insects where these proteins characteristically have molecular masses in the range of 50 kD to nearly 200 kD, and are concentrated in mature oocytes where they are the most abundant of the egg proteins (Postlethwait and Giorgi, 1985).

In nematodes, vitellogenin and vitellin have been described for *Caenorhabditis elegans* (Kimble and Sharrock, 1983; Sharrock, 1983, 1984) and for another free-living nematode of the genus *Dolichorhabditis* (Winter, 1992). Oocytes of *C. elegans* contain four vitellins (Sharrock et al., 1990), and those in *Dolichorhabditis* have three (Winter, 1992). They are the most abundant proteins in the oocyte and range from 82 kD to 188 kD (Sharrock et al., 1990; Winter, 1992).

No vitellogenic proteins have yet been described for any plant-parasitic nematode. The soybean cyst nematode, *Heterodera glycines,* has the greatest impact upon yield loss in soybean, *Glycine max,* of any pathogen in the United States (Kim et al., 1998). Females of *H. glycines* enlarge considerably as they feed and convert nutrients from the plant host into biochemicals necessary to support embryogenesis and reproduction. This significant physiological commitment involves the production of egg proteins, presumably including vitellins. The purpose of this work was to examine preparations of *H. glycines* for proteins that may be candidates for classification as soybean cyst nematode vitellins, and to partially characterize the proteins.

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Materials and Methods

Animals and rearing: Heterodera glycines race 3 were maintained in root culture consisting of sterile soybean cv. Kent root explants grown on 1.5% nutrient agar containing Gamborg's medium (Huettel, 1990). During development, the youngest females were white, and females proceeded through color changes to yellow and then brown as they matured (Young, 1992). Females were collected individually with a jeweler's forceps and dissecting stereomicroscope (×60). Collected females (yellow and brown) were stored at –20 °C. Eggs were harvested from individual yellow females by extruding the eggs using forceps pressure in a few drops of distilled water on a depression slide. Eggs were then cleaned from debris by rinsing with distilled water in a depression slide, allowing the eggs to settle, and then removing the rinse water with a micropipet. This was repeated twice, and eggs were stored at –20 °C. Bodies from which eggs were removed were stored separately at –20 °C. Second-stage juveniles (J2) were obtained from large-scale culture (Sardanelli and Kenworthy, 1997) by hatching from eggs in water over a 48-hour period, collecting by low-speed centrifugation (800*g*), and then storing at –20 °C.

Tissue extraction: Proteins from females, eggs, or J2 were extracted in 20 mM sodium phosphate, 400 mM NaCl, 1 mM phenylmethylsulfonylfluoride, pH 6.5, on ice. Extracts were centrifuged at 9,800*g* for 1 minute at room temperature, and aliquots of the supernatants were collected for storage at –20 °C.

Protein estimation: The microBCA protein assay was used according to the manufacturer's instructions (Pierce Chemical, Rockford, IL) and modified for the 96-well microtiter plate format. Standard curves were generated using bovine serum albumin, and colorimetric measurement was done at 560 nm.

Electrophoresis: Samples were combined with equal volumes of 20 mM TRIS-HCl, 2 mM ethylenediaminetetraacetic acid, 2 mM dithiothreitol, pH 8, containing 10% sodium dodecyl sulfate (SDS) and 0.02% bromophenol blue (all from Sigma Chemical, St. Louis, MO), and heated for 3 minutes at 95 °C. Treated samples were then applied to TRIS-glycine polyacrylamide gels (8 cm × 8 cm × 1 mm; 4–20% acrylamide/bisacrylamide; Novex, San Diego, CA). The running buffer was 25 mM TRIS, 192 mM glycine, pH 8.3 with 0.1% SDS (Novex). Protein bands were visualized by silver staining (Protostain, National Diagnostics, Atlanta, GA) following the manufacturer's instructions. Development times at 26 °C were typically 8 to 10 minutes, and staining intensity was linear from 30 to 300 ng total protein/gel lane. Standards used for molecular mass estimates were myosin (212 kD), α2-macroglobulin (170 kD), β-galactosidase (116 kD), transferrin (76 kD), and glutamic dehydrogenase (53 kD) (Pharmacia Biotech, Piscataway, NJ). Relative mobility, the ratio of the length of travel of individual protein bands to the length of travel of the sample marker dye, was used to estimate molecular mass. For relative quantification of bands, stained gels were recorded on an imaging system (Alpha Innotech, San Leandro, CA) used as a densitometer to convert bands to peaks. Peak areas were quantified using on-board integration software, and the relative abundance of each band was expressed as a percent of total peak areas integrated.

Data analysis: Means were compared by one-way ANOVA at the 95% confidence level with SAS/LAB (SAS Institute, Cary, NC).

Results

Extraction of female proteins: The mean total protein per female was greater in developmentally younger yellow females than in the developmentally older brown females (Table 1). Extracts of eggs obtained from yellow females contained a mean total protein content of 9.73 ± 2.46 ng/egg (Table 1). Extracts of bodies of yellow females from which eggs had been removed yielded 1.15 ± 0.16 ug/female, which was equal to 35.1% of the total protein per yellow female (Table

TABLE 1. Distribution of total protein in *Heterodera glycines* females.

Extract source	Protein per female or egg (mean ± sem)	Percent
Yellow females		
Whole	3.28 ± 0.48 ug	100
Eggs	9.73 ± 2.46 ng	(41.2)[a]
Body	1.15 ± 0.16 ug	35.1
"Removed" protein[b]	2.13 ug	64.9
Brown females	1.33 ± 0.16 ug	—

[a] Mean number of eggs extruded from females was 139 ± 24. At 9.73 ng/egg, the total egg protein/female was 1.35 ug or 41.2% of the whole female.
[b] Calculated difference between protein means of whole female and female body.

1). The females used in this study yielded a mean of 139 ± 24 extruded eggs per female. At 9.73 ng protein/egg, the amount of protein that could be accounted for in the egg complement per yellow female was 1.35 ug. This was 41.2% of the total protein recovered from an entire yellow female (Table 1).

Separation of female proteins: Electrophoresis of protein extract from yellow females yielded a number of stained bands (Fig. 1A, lane 2-yellow female extract), two of which stained especially intensely (Fig. 1A, lane 2, arrows). The two intensely stained bands migrated between the myosin (212 kD molecular mass) and α2-macroglobulin (170 kD molecular mass) markers (Fig. 1A, lane 1). Extracts from brown females yielded a slightly different electrophoretic pattern (Fig. 1A, lane 3-brown female extract). A band of high-molecular-weight material stained more intensely in the extract of brown females than in the extract of yellow females (Fig. 1A, lanes 2 and 3, band A), as

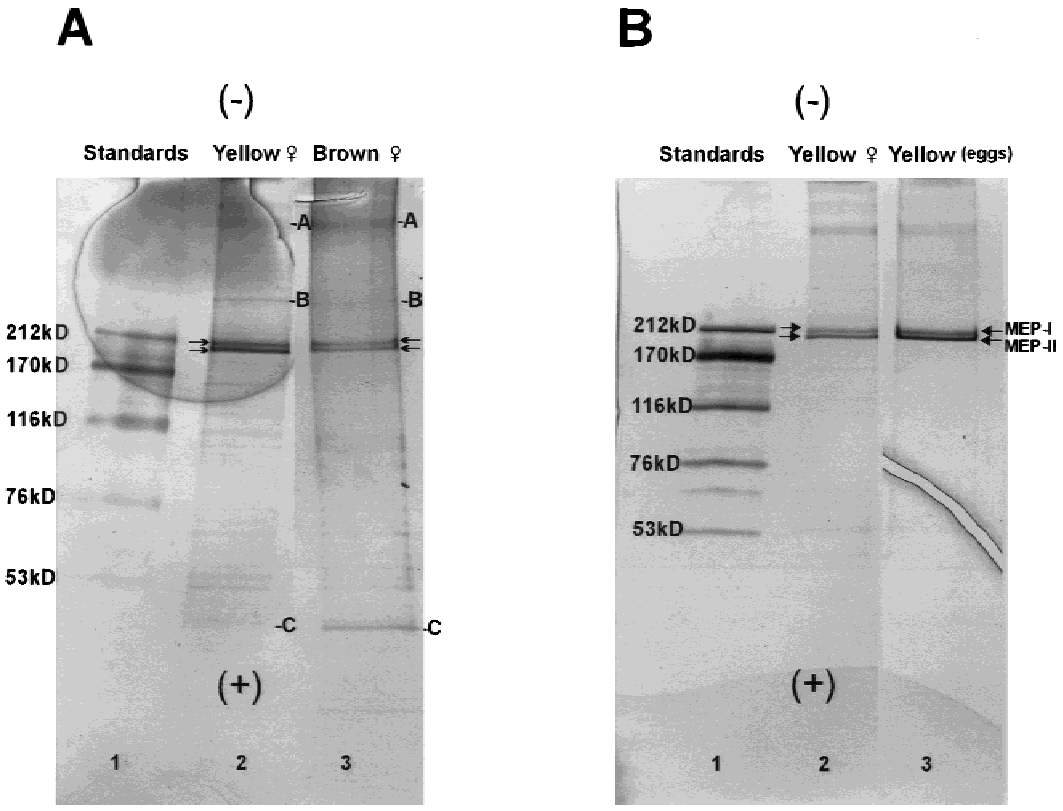

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *Heterodera glycines* extracts. Extracts were applied at 100 ng total protein per lane. Numbers next to lanes 1 indicate sizes of molecular mass markers. A) Yellow and brown female extracts. Samples were: lane 1—molecular mass markers; lane 2—extract of yellow females; lane 3—extract of brown females. Arrows indicate most intensely staining bands; thought to represent major egg polypeptides. A, B, and C indicate additional bands that change in staining intensity from yellow to brown females. B) Yellow female and yellow female egg extracts. Samples were: lane 1—molecular mass markers; lane 2—extract of yellow females; lane 3—extract of eggs from yellow females.

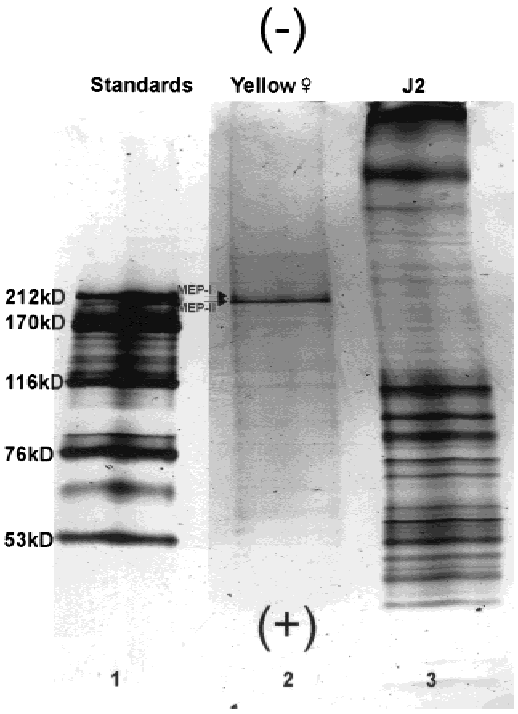

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *Heterodera glycines* yellow female and second-stage juvenile (J2) extracts. Samples were: lane 1—molecular mass markers; lane 2—extract of yellow females (50 ng total protein); lane 3—extract of J2 (125 ng total protein). Numbers next to lane 1 indicate sizes of molecular mass markers.

did a band of relatively low molecular weight (Fig. 1A, lanes 2 and 3, band C). Band B, migrating between band A and the 212 kD molecular mass standard, stained slightly more intensely in the yellow extract than in the brown extract (Fig. 1A; lanes 2 and 3). However, as in the extract from yellow females, the two most intensely staining bands in the brown extract migrated between the 212 kD and 170 kD molecular mass markers (Fig. 1A; lane 3, arrows). The relative staining intensities of the two bands marked by arrows was greater in the yellow female extract than in the brown female extract. Examination of extracts of eggs from yellow females showed an even more intense staining of the same two bands (Fig. 1B; lane 2-yellow female, arrows; lane 3-eggs from yellow females, arrows). Consequently, the proteins represented by these bands were named major egg polypeptide-I (MEP-I) and major egg polypeptide-II (MEP-II) (Fig. 1B; lane 3). Since brown females apparently contained MEP-I and MEP-II (Fig. 1A; lane 3, arrows), and the eggs of these females contain a high proportion of developing juveniles, J2 were examined for evidence of MEP-I or MEP-II. Neither polypeptide was detected with silver stain (Fig. 2; lane 3), although their absence cannot be conclusively established at this level of detection.

Partial characterization of MEP-I and MEP-II: Relative levels of MEP-I and MEP-II in various extracts were compared through quantification of the banding patterns by image analysis. Peak area integration showed that the combination of MEP-I and MEP-II accounted for 34.4 ± 1.5% of all detected proteins in yellow female electrophoretic separations (Table 2). Extracts of eggs from yellow females were enriched for the two MEPs, with a mean percent (55.8 ± 3.8%), significantly higher than that for yellow females (Table 2). In contrast, extracts of brown females had a significantly lower percent of total detected protein present in the two MEP bands (10.5 ± 3.2%) than either of the other extracts (Table 2). Major egg polypeptides were equally distributed between MEP-I and MEP-II in extracts of yellow females and eggs from yellow females (Table

TABLE 2. Major egg proteins (MEP) in *Heterodera glycines*.

Extract source	Total MEP[a] (mean ± sem)	MEP-I[a] (mean ± sem)	MEP-II[a] (mean ± sem)
Yellow females			
Whole	34.4 ± 1.5	17.2 ± 1.2	17.3 ± 0.3
Eggs	55.8 ± 3.8	28.1 ± 2.0	27.8 ± 3.0
Brown females	10.5 ± 3.2	6.4 ± 1.9	4.1 ± 1.3

[a] Data are expressed as percentages of peak areas for specific bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis relative to total integrated peak area. $N = 3$ for each sample mean.

2). In extracts of brown females, although total MEP was less than 50% of that in yellow females, the distribution of MEP between MEP-I and MEP-II was nearly equal (Table 2).

Molecular mass estimates for the *H. glycines* major egg polypeptides were 190 kD for MEP-I and 180 kD for MEP-II, placing these two *H. glycines* polypeptides in the size category occupied by the larger female-specific vitellins from *C. elegans,* YP170A (188 kD) and YP170B (186 kD), and *Dolichorhabditis* sp., VT1 (175 kD) (Table 3).

Discussion

Changes observed in soluble protein levels in *H. glycines* were functions of age and development, and included the most abundant proteins detected in extracts of *H. glycines* females and eggs, termed major egg polypeptides I and II (MEP-I and MEP-II). The significant loss of total extractable protein from females in the transition from yellow to brown stages most likely involved a number of factors. As females age, the rate of feeding should decrease, leading to less protein accumulation along with a slowing of egg development. A major contributing factor to the decline in yolk protein levels in insects is the use of such stored protein during embryonic and early development (Trewitt et al., 1992). In nematodes, at least some of the egg protein consumed during embryogenesis and juvenile development must be re-synthesized into less soluble forms, such as protein components of the juvenile cuticle. Also, changes in the female cuticle in anticipation of cyst formation may involve shifts in protein content from soluble to less soluble forms. Numerous other catabolic pathways must also contribute to the protein decline during the yellow female to brown female transition. If such a decline is indeed age- or development-related, then some proteins particularly associated with aging and development (e.g., egg yolk proteins, vitellins) will be noticeably affected. In *H. glycines,* total female protein declined by 2.5-fold from the yellow to brown stages. However, the amounts of at least two polypeptides specifically associated with the egg, MEP-I and MEP-II, declined by more than 5-fold during the same period. Of interest is an electrophoretic examination of *H. glycines* eggshell proteins (Kennedy et al., 1997) showing that all detected eggshell-specific proteins had molecular masses of 70 kD or less. This supports the argument that MEP-I and MEP-II are extracted from the yolk or other non-eggshell component. The age-related decline in the levels of the major egg polypeptides, at twice the rate of decline of the total protein pool, may indicate a selective use of MEP-I and MEP-II by the developing juvenile, although there is no direct evidence for this. However, J2 extracts did exhibit a complex electrophoretic banding pattern in which MEP-I or MEP-II could not be detected at the level of silver staining. If either MEP was transferred intact to developing juveniles, it may have been catabolized prior to the J2 stage. A definitive answer awaits additional (e.g., pulse-chase, Western blotting, etc.) experiments.

Over 40% of the total protein extracted from yellow females was found in the eggs, suggesting a major metabolic commitment

Table 3. Egg proteins of three species of nematodes.

Species	Egg protein	Molecular mass	Reference
Caenorhabditis elegans	YP170A	188 kD	Sharrock et al., 1990
	YP170B	186 kD	
	YP115	109 kD	
	YP88	83 kD	
Dolichorhabditis sp.	VT1	175 kD	Winter, 1992
	VT2	107 kD	
	VT3	82 kD	
Heterodera glycines	MEP-I	190 kD	present work
	MEP-II	180 kD	

to the production of egg proteins and especially vitellins. Such an investment is typical of reproductive female invertebrates (Chen et al., 1997; Spieth et al., 1991). In extracts of *H. glycines* eggs, over 50% of the protein resolved by SDS-PAGE was detected as two components, MEP-I and MEP-II. These data suggest that not only is the metabolism of young (yellow) female *H. glycines* targeted toward the production of egg proteins, but two of these proteins are produced at elevated levels relative to the remaining egg proteins. The proportion of total protein present as MEP-I and MEP-II was enriched in egg extract as compared with whole female extract, and the proportion of MEP as a percentage of total female protein was lower in brown females than in the younger yellow females. This suggests at least a temporal association between reduced MEP level and juvenile development. The two *H. glycines* MEPs have molecular masses similar to those of YP170A and YP170B from *C. elegans,* and VT1 from *Dolichorhabditis,* proteins identified as vitellins in those nematodes. Taken together, these observations suggest that MEPs are candidates for *H. glycines* vitellins. Definitive evidence requires further chemical characterization (e.g., carbohydrate and lipid analyses) and physiological data (e.g., site and regulation of production).

Vitellogenin production in *C. elegans* is under the control of sex-, stage-, and tissue-specific factors (Blumenthal et al., 1984; Kimble and Sharrock, 1983; Sharrock, 1983, 1984; Trewitt et al., 1992) and is associated with a family of six genes (*vit-1–vit-6,* Spieth et al., 1991), implying a complex set of regulatory mechanisms. The smaller vitellins of both *C. elegans* (YP-115, YP-88, Spieth et al., 1991) and *Dolichorhabditis* (VT-2 and VT-3, Winter et al., 1996) are products of *vit-6.* Since vitellin-like polypeptides of less than 180 kD were not detected in *H. glycines,* potentially interesting genetic differences may exist between the plant parasite and the two free-living nematodes. Use of such interspecific information in the characterization of vitellin and vitellogenin production in *H. glycines* is necessary to elucidate analogous mechanisms in this species, and to identify molecular targets for novel control agents. Inhibition of vitellogenin production, for example, would be a clearly powerful means of *H. glycines* control.

Comparisons among nematode species are also important, as vitellins represent a fast-evolving group of proteins and should be useful for establishing phylogenetic relationships among closely related species (Winter et al., 1996). Because the main function of vitellin is to provide a source of amino acids for use during embryogenesis and early development (Byrne et al., 1989), there is little selective pressure, leading to highly divergent vitellin amino acid sequences among vertebrates and invertebrates (Byrne et al., 1989; Winter et al., 1996). However, some structural features and amino acid composition have been conserved from vertebrates through nematodes (Spieth et al., 1991). Indeed, vitellin amino acid sequences are more similar between nematodes (i.e., *C. elegans*) and vertebrates than between nematodes and insects (Trewitt et al., 1992). Vitellins from vertebrates, insects, and nematodes share a common ancient ancestor (Chen et al., 1997; Trewitt et al., 1992), and vitellogenin gene expression may also have parallels among these diverse taxa (Wahli, 1988). Such relationships are clearly important for taxonomic studies but are equally important in evaluating the relevance of biochemical and developmental data obtained from *C. elegans* to studies of parasitic species (Winter, 1992; Winter et al., 1996), including *H. glycines.*

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