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FMRFamide-like Immunoactivity in *Heterodera glycines* (Nemata: Tylenchida)

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Abstract: Material antigenically related to the neuromodulatory peptide FMRFamide was detected and examined in preparations of the soybean cyst nematode, *Heterodera glycines,* and in the free-living nematodes *Caenorhabditis elegans* and *Panagrellus redivivus.* FMRFamide-related peptides were quantified by an enzyme-linked immunosorbent assay. Specific activities were remarkably similar among all of the vermiform members of the three species. FMRFamide-related peptide immunoactivity was present in both sexes and all stages of *H. glycines* examined. The highest specific activity was present in second-stage juveniles and in males, and the lowest in white and yellow females. Total FMRFamide-related peptide level per individual was highest in brown females, with 90% of the activity associated with the eggs. Peptide levels in these eggs and in second-stage juveniles were comparable and increased in adults, especially in females. Chromatographic analysis of FMRFamide-related peptide preparations from *H. glycines* juveniles, *C. elegans,* and *P. redivivus* revealed distinct qualitative differences between the infective plant parasite and the free-living nematodes.

Key words: Caenorhabditis elegans, ELISA, FaRP, female aging, FMRFamide, *Heterodera glycines,* juvenile development, nematode, *Panagrellus redivivus,* peptide, RP-HPLC, soybean cyst nematode.

Neuroregulators, including neuropeptides, are involved in essentially all developmental and metabolic processes in vertebrates and invertebrates (Shaw, 1996) and are the subjects of research as potential leads to the development of veterinary pharmaceuticals (Brownlee et al., 1996; Geary et al., 1995) and invertebrate pest control agents (Masler et al., 1993). Given such extensive involvement in other animals, it is reasonable to expect that neuropeptides play important regulatory roles in nematodes (Davis and Stretton, 1995; Shaw, 1996). Indeed, immunohistochemical screens have revealed extensive antigenic homologies in nematodes to a variety of vertebrate and invertebrate anti-peptide antisera (Brownlee et al., 1994; Sithigorngul et al., 1990, 1996). However, until recently, neuropeptides as a molecular group have received comparatively little attention in nematodes (Rand and Nonet, 1997). Research on nematode neuropeptides has begun with a focus on a family of neuropeptides, characterized by an arginine-phenylalanine-amide C-terminal motif. The first reported peptide in this family had the sequence phenylalanine(F)-methionine(M)-arginine(R)-phenylalanine(F)-amide (named FMRFamide) and was shown to have cardioacceleratory properties (Price and Greenberg, 1977). Subsequently, similar peptides ranging in size from 4 to 36 residues have been described in all animals examined and exhibit some form of neuromuscular activity (Brownlee et al., 1996; Demichel et al., 1993; Holman et al., 1991). As a group, these peptides are referred to as FaRPs (*F*MRF*a*mide-*R*elated *P*eptides). FaRPs have been isolated from animal-parasitic nematodes and free-living nematodes (Brownlee et al., 1996), and their roles in neuromuscular activity have been examined. There is much current research on the genetics and physiology of FaRPs in free-living and animal-parasitic nematodes (Brownlee et al., 1996; Cowden and Stretton, 1995; Rosoff et al., 1993; Schinkman and Li, 1992), and their potential as leads to new nematode control agents (Geary et al., 1995).

Apart from immunocytochemical studies (Atkinson et al., 1988), nothing is known about this group of bioactive peptides in plant-parasitic nematodes. The purposes of

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this research were to (i) detect and measure FaRP immunoactivity in the soybean cyst nematode, *Heterodera glycines* Ichinohe, (ii) examine this activity during the development of and in the two sexes of *H. glycines,* and (iii) present qualitative and quantitative comparisons of FaRP immunoactivity between *H. glycines* and free-living nematodes.

Materials and Methods

Animals: Panagrellus redivivus and *Caenorhabditis elegans* were reared at 22 °C in medium containing yeast extract (30mg/ml; Difco, Detroit, MI), soy peptone (30 mg/ml; Sigma Chemical, St. Louis, MO), dextrose (10 mg/ml; Sigma), hemoglobin (0.5 mg/ml; Sigma), sitosterol (10 µg/ml; Sigma), and Tween-80 (0.5 mg/ml; Sigma) (Chitwood et al., 1995). All sampling and measurements with the free-living nematodes were done with cultures not sorted by age or sex. An isolate of *H. glycines* race 3 was maintained and increased on *Glycine max* cv. Essex for the collection of large quantities of females, eggs, and second-stage juveniles (J2) in a whole-plant culture system with soil moisture control (Sardanelli and Kenworthy, 1997). Females were collected according to color stages of maturation. The youngest females were white and proceeded through yellow to brown as they matured (Young, 1992). Second-stage juveniles were harvested after a 24-hour egg hatch at room temperature on modified Baermann funnels. Eggs were collected from brown cysts (Sardanelli and Kenworthy, 1997).

For some collections (e.g., males), *H. glycines* race 3 was grown in root culture on soybean cv. Kent root explants maintained on 1.5% agar containing Gamborg's B-5 medium (Huettel, 1990). Nematodes were washed from the surface of the agar cultures with repeated rinsing over a 45-µm-pore sieve with tap water from a 500-ml widespout wash bottle. The washings were placed on Baermann funnels and collected after 24 hours. Males and J2 were separated by hand, and extraneous materials were removed from individual female, egg, J2, or male collections by hand-picking with a pipet. Concentrations of *H. glycines* life stages in suspensions were determined by counting three 1-ml aliquots at ×40. Samples of vermiform nematodes from each culture were measured for length and diameter with an ocular micrometer and average volumes were calculated, assuming a cylindrical shape.

Extractions: Nematodes collected from plant, root, or liquid culture were washed at least 5 times by gentle agitation in HPLC-grade water (distilled and filtered through a reverse osmosis system). Following each wash, nematodes were allowed to settle to the bottom of the tube before decanting. Final washes were assayed by immunoassay and protein assay (see below) to ensure that no FaRP immunoactivity or other proteins were present. Also, *C. elegans* and *P. redivivus* culture media were examined for FaRP immunoactivity (none was detected). After the final water wash, the settled nematodes were suspended in 20 volumes of 50% acetonitrile (CH_3CN; HPLC grade, Fisher Scientific, Springfield, VA) acidified with trifluoroacetic acid (TFA; Aldrich Chemical, Milwaukee, WI) to 0.1% TFA. The suspensions were incubated at 4 °C for 7 to 10 days with regular shaking. Suspensions were centrifuged at 10,000g for 20 minutes and the supernatants were collected, divided into aliquots, dried under vacuum (SpeedVac apparatus, Savant Instruments, Farmingdale, NY), and stored at −20 °C. To confirm that the bulk of FaRP immunoactivity was in the extract supernatants, the pellets were also examined. The pellets, containing the nematodes, were re-extracted by sonication in acetonitrile-TFA with a micro-tip sonicator (Sonifier, Branson Ultrasonics, Danbury, CT) set at 50% maximum power with 1-second pulses for 30 seconds. Sonication was repeated 2 times, and samples were examined under a dissecting microscope (×40) to confirm that nematodes had been disrupted. The samples were then incubated at 4 °C overnight, and the sonication was repeated. These extracts were centrifuged as above and the supernatants examined for immunoactivity. The level of FaRP immunoactivity detected in the re-extracted pellet

preparations was less than 4% of the level measured in the original extract supernatant. Consequently, original extract supernatants were used for all studies and pellets were discarded.

Immunoassay: The FaRP enzyme-linked immunosorbent assay (ELISA) was described by Kingan et al. (1997). The primary antiserum was rabbit polyclonal anti-FMRFamide (Marder et al., 1987; provided by Eve Marder). Secondary antibody (goat anti-rabbit IgG, Fc) was obtained from American Qualex (La Mirada, CA). Synthetic peptide ligands, including FMRFamide, were from Sigma. The FMRFamide-horseradish peroxidase (FMRFa-HRP) conjugate was prepared according to Kingan et al. (1997) and stored at −50 °C. Standard curves were constructed with FMRFamide (2-500 fmole/well), data were analyzed by linear regression, and the level of antigenic activity was expressed as FMRFamide equivalents (Feq). The IC_{50} for FMRFamide was typically 0.5–1.0 fmole Feq/µl.

Protein assay: The microBCA protein assay was used according to the manufacturer's instructions (Pierce Chemical, Rockford, IL), modified for the 96-well microtiter plate format.

Chromatographic fractionation: Samples were fractionated by reverse-phase chromatography on a C_{18} column (DeltaPak, 5 µm, 300Å, 7.8 × 300 mm, Waters Chromatography, Milford, MA) equipped with a guard column (Sentry, DeltaPak C_{18}, 15 µm, 300Å, 3.9 × 20 mm, Waters). The mobile phase was 5% CH_3CN in 0.1% TFA for 20 minutes followed by a linear gradient 5%–75% CH_3CN in 0.1% TFA for 70 minutes. Flow rate was 1 ml/min. Fractions were collected and aliquots were dried in the SpeedVac, then stored at −20 °C. Aliquots were assayed for total protein and FaRP immunoactivity.

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

Results

Immunodetection Several FaRP sequences were screened and, not surprisingly, the ELISA was most sensitive in detecting FMRFamide (IC_{50} = 0.7 nM; Table 1). The presence of arginine-phenylalanine at the amidated C-terminus was essential for immunoactivity, as a modified arginine (D-amino acid) rendered the peptide undetectable (IC_{50} > 100 µM). Modifications of the peptides not involving the C-terminal arginine-phenylalanine had varying effects upon detectability (Table 1). Nevertheless, the ELISA clearly detected a variety of FaRP sequences, and it is reasonable to presume that total immunoactivity in nematode extracts represented a combination of immunoactivities contributed by various sequences.

FaRP immunoactivity in H. glycines and free-living nematodes: Inter-species comparisons were done using similar morphological forms, i.e., vermiform stages. No differences ($P < 0.05$) were detected among the mean specific activities determined for samples of *P. redivivus* (33.5 ± 7.6 fmole Feq/µg), *C. elegans* (55.6 ± 10.1 fmole Feq/µg), or from collections of *H. glycines* J2 (52.6 ± 12.4 fmole Feq/µg).

Distribution of FaRP immunoactivity and total protein in H. glycines developmental stages: FaRP immunoactivity was present in both sexes and at all developmental stages of *H. glycines* examined. Mean specific activities of selected stages (Fig. 1) ranged from 2.4 ± 1.0 fmole Feq/µg for females to 52.6 ± 12.4 fmole Feq/µg for J2. Males and eggs had specific activities of 26.0 ± 5.5 and 12.1 ± 1.3 fmole Feq/µg, respectively. The means for eggs, J2, and males were not different ($P < 0.05$), but all were greater than the mean specific activity for females. In contrast to

Table 1. IC_{50} values for selected FaRPs sequences.

Sequence	IC_{50}
FMRFa	0.7 nM
NacFnLRFa	3 nM
dFMRFa	6 nM
FLRFa	11 nM
FdMRFa	75 nM
pEDPFLRFa	200 nM
FMdRFa	>100 µM

Peptides (antigens) were tested separately in competition with the FMRFa-HRP conjugate.

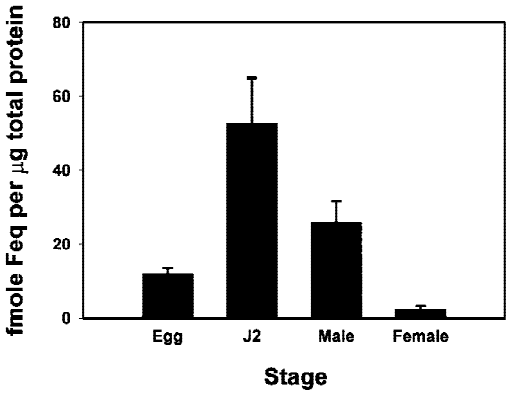

FIG. 1. Specific FaRP immunoactivity in *Heterodera glycines*. Parallel extract aliquots were assayed for total Feq and protein. Each bar represents the mean specific activity (fmole Feq per μg total protein) ± SEM of 4 to 6 separate extractions. J2 = second-stage juveniles.

specific activities, where only females differed from the other stages examined, mean protein levels per individual were different for all stages (Fig. 2). By far, the greatest amount of protein per individual was in females (1,420 ± 250 ng/female) followed by males (20 ± 5 ng/male). The lowest levels were in J2 (1.1 ± 0.4 ng/J2) and eggs (3.1 ± 0.9 ng/egg). FaRP levels per individual fell into several groups. Eggs and J2 (Fig. 3A) each contained the same average FaRP level (0.04 ± 0.01 fmole Feq/egg, 0.05 ± 0.01 fmole Feq/J2). Males had a 10 to 12-fold higher FaRP level (Fig. 3A; 0.54 ± 0.24 fmol Feq/male) than eggs or J2—a level similar to that observed in white and yellow females (Fig. 3B; 0.86 ± 0.24 fmol Feq/female). Brown cysts had by far the highest FaRP level

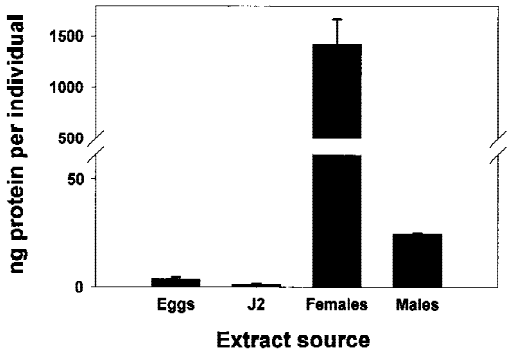

FIG. 2. Total protein in *Heterodera glycines*. Data represent the mean ± SEM for 4 to 6 separate extractions. J2 = second-stage juveniles.

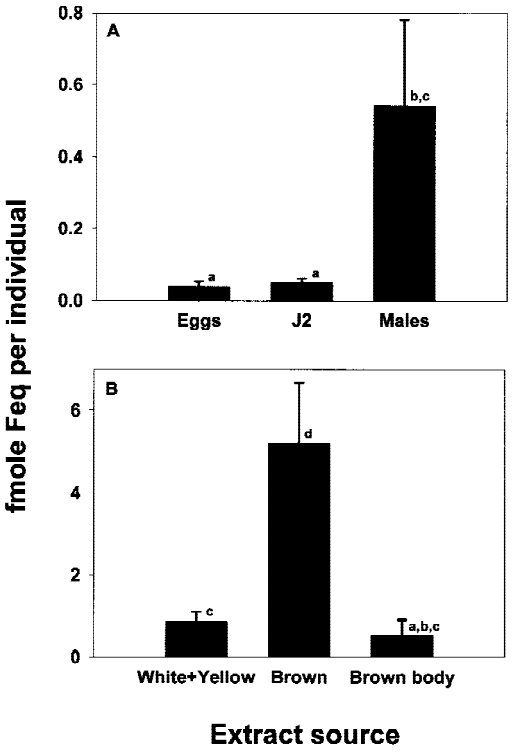

FIG. 3. Total FaRP immunoactivity in *Heterodera glycines*. A) Eggs from brown females, second-stage juveniles (J2), and males. B) Combined white and yellow females, brown females, and brown female bodies (from which eggs were removed). Data represent the mean total Feq per individual ± SEM for 3 to 7 separate extractions. Means not sharing the same letter were significantly different ($P < 0.05$).

(Fig. 3B; 5.20 ± 1.48 fmol Feq/female) detected in any stage, 6-fold greater than the less mature (white or yellow) females and 10-fold greater than males. Interestingly, when eggs were forcibly extruded from brown cysts and the remaining bodies rinsed, and assayed by ELISA, the total detectable FaRP level was reduced by 90% (0.53 ± 0.38 fmol Feq/brown body) compared with the level measured in whole brown cysts. The mean FaRP level in brown bodies was similar to the levels observed in eggs, J2, males, and younger (white and yellow) females. Comparison of total FaRP levels among the developmental types examined in *H. glycines* indicated that ''mature'' stages (brown cysts and males) had higher levels than ''immature'' stages (eggs and J2). In addition, the mean FaRP level for

brown cysts was greater than that of white and yellow females. This caused us to look more closely at changes in FaRP levels during female development.

FaRPs and total protein levels during female aging: Examination of white, yellow, and brown stages separately revealed changes in FaRP levels during development from white and yellow to the brown stage (Fig. 4). The total amounts of Feq per individual (Fig. 4, black bars) were not statistically different between white and yellow females (0.60 ± 0.50 fmole Feq/female and 1.01 ± 0.34 fmole Feq/female, respectively), but Feq per female did increase significantly from yellow to brown (5.28 ± 1.48 fmole Feq/female). Total protein per individual (Fig. 4; white bars) remained relatively constant (1.16 ± 0.73 µg/white female, 2.01 ± 0.36 µg/yellow female, 0.89 ± 0.24 µg/brown cyst). The significant increase in specific activity in brown cysts (Fig. 4 inset, hatched bars; 6.23 ± 1.92 fmole Feq/µg) over white (0.43 ± 0.11 fmole Feq/µg) and yellow (0.48 ± 0.09 fmole Feq/µg) females was due solely to the increase in fmole Feq. Also, these cysts had by far the highest total FaRP levels among all stages examined (Fig. 3). However, since the highest specific activity was observed in J2 (Fig. 1; 10-fold greater than brown cysts, 2-fold greater than males), this stage was chosen for further examination of FaRP activity in *H. glycines*.

Chromatography: Extracts of *H. glycines* J2, *C. elegans,* and *P. redivivus* were compared by reverse phase chromatography (Fig. 5). Each extract yielded a number of immunoactive fractions eluting between 30% and 50% CH_3CN on the gradient. All three species showed significant activity in two fractions eluting at 54-55 minutes (near 35% CH_3CN; Fig. 5A, B, C). This activity accounted for more than 50% of all FaRP immunoactivity observed from *H. glycines* (Fig. 5C) and less than 25% of total FaRP immunoactivity detected from either *P. redivivus* (Fig. 5A) or *C. elegans* (Fig. 5B). *Panagrellus*

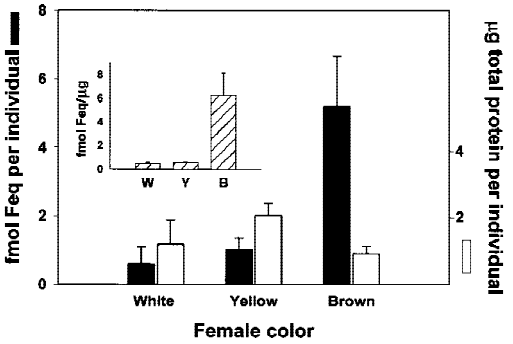

FIG. 4. Total protein and FaRP immunoactivity in *Heterodera glycines* females during development. Females were divided into three groups (white, yellow, brown). Parallel extract aliquots of each were assayed for total protein and Feq. Data represent means ± SEM for 3 to 5 separate extractions. Black bars, fmole Feq/female; white bars, µg total protein/female; inset, hatched bars, specific activities (fmole Feq per µg protein) for white (w), yellow (y), and brown (b) females.

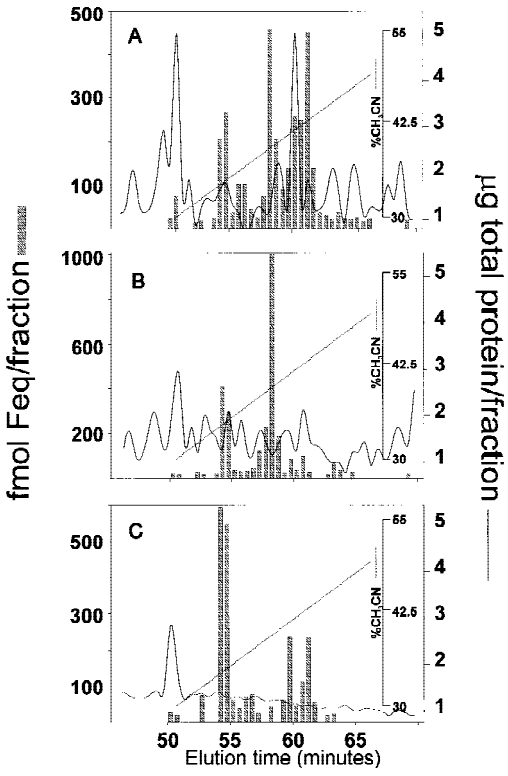

FIG. 5. FaRP immunoactivity and total protein chromatographic profiles of *Panagrellus redivivus, Caenorhabditis elegans,* and *Heterodera glycines.* Aliquots of each fraction were assayed for total protein and Feq. The solid line represents total protein (µg) per fraction. Vertical bars represent total fmole Feq per fraction. The slanted line in each panel indicates the portion of the acetonitrile (CH_3CN) gradient between 30% and 55%. Flow rate was 1 ml/minute. A) *P. redivivus,* 195 µg total protein applied to the column. B) *C. elegans,* 105 µg. C) *H. glycines,* 73 µg.

redivivus and *C. elegans* each had a prominent peak of activity at 58 minutes (41% CH_3CN; Fig. 5A, B), one of two quantitatively major peaks in *P. redivivus* (the other at 61.5 minutes, 43% CH_3CN). The 58-minute peak was the single largest peak in *C. elegans*. Only a trace amount (< 1%) of total FaRP immunoactivity detected from *H. glycines* was present in the fraction that eluted at 58 minutes (Fig. 5C).

Discussion

The results presented in this study demonstrate that FMRFamide-like material is present in and can be extracted and quantified from various developmental stages of *H. glycines,* as well as from the free-living nematodes *C. elegans* and *P. redivivus*. This is the first such report for any plant-parasitic nematode. In comparing *H. glycines* with the free-living nematodes, one of the most significant observations was that specific activities were the same for the three species, including both J2 and males of *H. glycines*. In contrast, total protein and FaRP levels appeared simply to approximate to relative sizes. Based upon calculated average volumes per nematode of 3.8 nl *(P. redivivus),* 1.2 nl *(C. elegans),* 1.1 nl (*H. glycines* males), and 0.2 nl (*H. glycines* J2), *H. glycines* males were 5.5× larger than J2. Males had 18× more protein and 11× more FaRP immunoactivity than J2. Individual *C. elegans* and *P. redivivus* were 6 and 19× larger, respectively, than *H. glycines* J2. Protein and FaRP levels (data not given) were 8× and 10× greater in *C. elegans* and 50 × and 55 × greater in *P. redivivus* than in *H. glycines* J2. In all comparisons among the vermiform stages, protein content and FaRP levels varied relative to size, but specific FaRP immunoactivity was constant, suggesting that FaRPs have equal physiological importance in all of the mobile nematodes examined, regardless of size or species. All of the vermiform stages were physically active, indicating a dependence upon somatic muscles for mobility and perhaps also upon muscles involved with feeding and digestion. Since FaRPs are associated with muscular regulation (Brownlee et al., 1996), an argument can be made that the FaRP level relative to total protein (i.e. specific activity) is an indicator of the importance of muscular activity.

In *H. glycines,* the total FaRP level in brown cysts was the highest of any of the stages examined, followed by the levels observed in white and yellow females, and males. After eggs were removed from brown females and the remaining body examined, only about 10% of the intact female activity (fmole Feq) remained. Thus, the majority of the activity detected in brown cysts was associated with the eggs. Although we did not measure total Feq in eggs from either white or yellow females, the fact that whole female FaRP levels in white and yellow females were so much lower than in brown females logically suggests that the eggs observed in white and yellow females contained significantly less FMRFamide-related peptide than did the eggs of brown cysts.

As juveniles develop and become ready to hatch, they become more active, suggesting a requirement for increased muscular activity and the neuromodulators involved. The sequential development of oocytes (Bird and Bird, 1991; Schedl, 1997; Triantaphyllou and Hirschmann, 1962) and retention of a large proportion of mature eggs (i.e. with juveniles) by *H. glycines* results in the accumulation of unhatched juveniles within the female. The increase in total Feq from younger females (white, yellow) to older (brown) females perhaps reflects changes in number and development of the juveniles. Since FaRPs are associated with neuromuscular activity, and the vermiform juvenile is a mobile stage, an increasing FaRP level associated with juveniles is not surprising.

The residual (10%) FaRP immunoactivity observed in brown cysts from which eggs had been removed may have been due to incomplete removal of eggs but perhaps, at least in part, it was also associated with neurons serving remaining abdominal musculature such as that of the uterus or oviduct. Despite having the highest FaRP level detected in any *H. glycines* stage, females had the lowest specific activity. This was clearly not surprising since females also had the

highest whole-body protein level. What is not clear is the source of the protein. Attention obviously focused on the eggs which, during development, act as protein sinks. However, not all protein can be accounted for in the eggs. Eggs contained 90% of total FaRP Feq in whole females. If specific activities were the same for eggs and whole females, one would expect that eggs contained 90% of the total protein as well. However, eggs had a 5× higher specific activity than whole females. Thus, a significant amount of extractable protein in whole females is not part of the eggs. This protein may be associated with the abdominal organs mentioned above, body wall musculature, and sites of protein production such as the intestine.

Distinct differences in the distribution of FaRP immunoactivity among *H. glycines, P. redivivus,* and *C. elegans* were observed by chromatographic analysis. Numerous immunopositive components were present in all extracts. Although a number of these components were present in all three species, based upon elution times, species-specific profiles of FaRP immunoactivity were generated. It is important to note that multiple FaRP sequences have been observed in all nematode species examined. Some FaRP amino acid sequences are shared by two or more species, but most of the sequences confirmed (biochemically) or predicted (genetically) appear to be unique to an individual species (Brownlee et al., 1996; Cowden and Stretton, 1995; Geary et al., 1995; Rosoff et al., 1993). Since the antiserum used in the present study detected, with varying degrees of efficiency, a number of FaRP amino acid sequences, the production of complex chromatographic ''fingerprints'' was not surprising. While each of the three species examined presented a unique chromatographic profile, prominent aspects of the three profiles distinguished the free-living species from *H. glycines.* For example, at a chromatographic elution time when large amounts of FaRP immunoactive material eluted in *P. redivivus* and *C. elegans* preparations (58 minutes), only trace amounts eluted in *H. glycines* J2 preparations. Immunoactivity that marked the major FaRP component of the *H. glycines* parasite accounted for less than one-quarter of all detected activity in either of the free-living species. Such differences in the relative abundance of FaRP immunoactivity in the chromatographic profiles indicate biochemical differences between the free-living and plant-parasitic nematodes. Also, the protein chromatographic profiles of the free-living species were strikingly more complex than that of *H. glycines.* These observations need to be expanded through the survey of other species and the use of additional antisera.

It is interesting to note that, in an immunocytochemical study, Atkinson et al. (1988) observed FMRFamide-like activity throughout the nervous systems of *C. elegans, P. redivivus,* and *H. glycines* J2. While activity was detected in similar locations within the nervous systems of all three species, important differences were observed. Neurons innervating the pharynx of *H. glycines* and neural tissue near the ventral nerve cord anterior to the anus contained FMRFamide-like material, but none was detected in the corresponding locations of either free-living species. The precise nature of these differences, and those observed in the present study, cannot be described until biochemical characterizations are done. Nonetheless, immunological evidence obtained by these two entirely different methods indicates that important differences exist among FaRPs in free-living nematodes and in *H. glycines.* It will be of interest to find out if these differences are at all related to parasitic characters.

FaRP immunoactivity is present in all developmental stages in *H. glycines,* but the high specific activity in J2 and the clean chromatographic profile make this the stage of choice for biochemical characterization of *H. glycines* FaRPs. Isolation and purification programs are currently under way in our laboratory.

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