Amino Acids from *Heterodera gylcines*[1]

SHEILA AIST AND ROBERT D. RIGGS[2]

Abstract: Amino acids emitted and extracted from surface-sterilized larvae and adults of *Heterodera glycines* were identified by paper chromatography and quantitatively analyzed by column chromatography. Five amino acids (alanine, aspartic acid, glutamic acid, glycine and serine) were emitted by *H. glycines* larvae and eight others (asparagine, glutamine, leucine/isoleucine, lysine, methionine sulfoxide, threonine, tyrosine, valine/methionine) were found in extracts from crushed larvae.

In addition to the amino acids emitted or extracted from larvae, four others were emitted by adults (γ-aminobutyric acid, histidine, phenylalanine, and proline). Four different amino acids (arginine, cystathionine, hydroxyproline, and ornithine) were found only in the extract from crushed adults. Greater quantities of alanine, aspartic acid and glycine were emitted than could be detected in nematode extracts suggesting selective emission.

Subsamples of nematode populations were taken from growing plants 19, 26, 33, and 40 days after inoculation and extracted to determine whether changes in specific amino acid content correlated with aging. Proline content shifted most, increasing from 4.1% to 21.5% of the total amino acid complement from the 19th to the 40th days.

The chemical identification of secretions, excretions, and extractions from plant-parasitic nematodes has been delayed by the difficulty in obtaining sufficient numbers of nematodes to yield measurable quantities of chemicals.

Myers and Krusberg (6) studied the amino acids discharged by three plant parasitic nematodes, *Ditylenchus dipsaci*, (Kuhn) Filipjev, *Pratylenchus penetrans* (Cobb) Chitwood & Oteifa, and *Meloidogyne incognita* (Kofoid & White) Chit. Aspartic acid, glutamic acid, serine, glycine, ornithine, threonine, α-alanine, methionine sulfoxide, asparagine, lysine, arginine, and isoleucine/leucine were discharged by *D. dipsaci* and *M. incognita, P. penetrans* discharged all except asparagine, lysine, arginine and isoleucine/leucine.

Changes in free amino acid composition of *Heterodera rostochiensis* Wollenweber, were studied by Smith and Ellenby (10). As the female matured from white to yellow and then to brown cyst, glutamic acid, aspartic acid, and serine increased. Glycine, proline, valine and leucine decreased and threonine, alanine, methionine and isoleucine approached zero.

Resistance to *Heterodera glycines* Ichinohe, was found in *Glycine max* (L.) Merr. (var. 'Peking') (9) and was subsequently transferred to a commercially acceptable variety, 'Pickett.' The chemcial nature of this resistance was not known, and studies were initiated to elucidate this subject. The purpose of the present study, part of a larger project to investigate the chemistry of resistance to soybean-cyst nematodes, was to determine the amino acids emitted and retained by *H. glycines.*

MATERIALS AND METHODS

A culture of *H. glycines* maintained on soybean, *Glycine max* (var. 'Lee'), in a greenhouse soil-bed was the source of nematodes for all studies. Cysts were separated from the soil and roots by roiling and sieving and then blended as described by Riggs and Hamblen (8). For direct hatch and recovery of larvae the blended material was placed on a Baermann funnel and the larvae drawn off daily and stored at 4 C for 2–4 days. Some

Received for publication 26 February 1969.

[1] This investigation was supported by CSRS Grant 616-15-1.

[2] Formerly Graduate Assistant, Department of Plant Pathology, University of Arkansas, presently Research Specialist, Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin 53706, and Plant Pathologist, Department of Plant Pathology, University of Arkansas, Fayetteville, Arkansas 72701, respectively.

changes in physiology may occur in storage, therefore storage time was kept at a minimum. To produce mature females of known age, the blended cyst material was used directly to inoculate young soybean plants growing in white quartz sand. After 19–40 days the mature females were separated from the plants and sand by the roiling and sieving technique and manually separated from roots and organic debris.

Nematodes to be incubated were surface sterilized by a modification of the technique used by Myers and Krusberg (6). The nematodes were placed in a 60-ml Buchner funnel with a medium porosity fritted glass filter and treated as follows: (i) 20 ml 0.1% penicillin G plus 20 ml 0.1% streptomycin sulfate for 45 min; (ii) 40 ml 0.05% hexadecyltrimethyl-ammonium bromide for one minute; (iii) rinsed with 40 ml sterile H_2O; (iv) 40 ml 0.5% chlorhexidine acetate[3] for six min; (v) 5 rinses with 40 ml sterile, deionized H_2O. Bacteriologically filtered air was used to force the solutions through the filter.

Surface sterilized larvae (50,000–100,000/sample) were incubated in the same Buchner funnel on a wrist-action shaker at 27–29 C for 42–60 hr in sterile, deionized water. Adults were incubated in 1% glucose, either in tubes in a water-bath shaker or in Petri dishes, for 24 hr at 28 C. An aliquot of each incubation mixture was placed on sterile nutrient agar to check the level of bacterial contamination. The incubation solution was forced through the fritted filter, evaporated to dryness in a Rinco flash evaporator and analyzed qualitatively or quantitatively for the presence of amino acids. Some batches of nematodes from the incubation tests were frozen and stored for later extraction while other batches were extracted immediately. Fresh or frozen nematodes from the incubation studies were ground with 3–6 ml of 1% picric acid. The precipitate was removed by centrifuging at 170 g for 15 min. The supernatant was passed through a column of Dowex 2 × 10 Cl^- resin and followed by 15 ml 0.2N HCl (11). Both fractions were combined, evaporated to dryness and analyzed for amino acids.

Some nematodes were freeze-dried with no incubation. The freeze dried material was weighed, ground with a small amount of 1% picric acid to a pasty consistency with an agate mortar and pestle, centrifuged to remove the precipitate, and the picric acid removed (11). Microscopic examination of the material in the mortar revealed that grinding broke many eggs and larvae. In an attempt to extract the amino acids from adults without extracting eggs or larvae, the adults were crushed in the mortar rather than ground. Crushing the adults broke few eggs and larvae. The mixture was processed as in the preceding paragraph, and the resulting precipitate was ground and also processed.

Qualitative analyses of amino acids were made by paper chromatography. The materials from the incubation or the extraction solutions were dissolved in 80% aqueous ethanol and desalted by the method of Plaisted (7). The eluates were evaporated to dryness, redissolved in 10% propanol, and spotted on 46 × 57 cm Whatman No. 1 chromatography paper. Amino acids were separated by two-dimensional descending chromatography by the method of Grable, *et al.* (5), using phenol-water (4 : 1 V/V) and butanol-acetic acid-water (4:6 V/V/V). Papers were thoroughly dried in an oven after each development. The papers were dipped in 1% ninhydrin in 95% ethanol and heated to 100 C for 15 min for detection of amino acids.

Column chromatography was used for quantitative analyses. The dry incubate or deproteinized extract from incubated nema-

[3] This material was supplied as Hibitane® by Ayerst Laboratories, Inc., New York, N. Y.

TABLE 1. Amino acids in incubation fluids and extracts from *Heterodera glycines* separated by paper chromatography. (+ indicates a spot was detected).

Amino Acid	Larvae		Adults	
	Incubate	Extract	Incubate	Extract
Alanine	+	+	+	+
Arginine				+
Asparagine		+	+	+
Aspartic Acid	+	+	+	+
Cystathionine				+
γ-Amino Butyric Acid			+	+
Glutamic Acid	+	+	+	+
Glutamine		+	+	+
Glycine	+	+	+	+
Histidine			+	+
Hydroxyproline				+
Isoleucine, Leucine		+	+	+
Lysine		+	+	+
Methionine sulfoxide		+	+	+
Ornithine				+
Phenylalanine			+	+
Proline			+	+
Serine	+	+	+	+
Theronine		+	+	+
Tyrosine		+	+	+
Valine, Methionine		+	+	+

todes was dissolved in 2 ml sodium citrate buffer pH 2.2 and was layered on a 50 cm column of Bio-Rad MSQ 50 resin. Amino acids were eluted with .2M sodium citrate buffers pH 3.28 and 4.26. The flow rate was 33 ml per hour. Forty drops of eluate per tube were collected with a Beckman Fraction Collector. One ml of ninhydrin solution (11) was added to each tube, the mixture was heated in a water bath at 100 C for 15 min, cooled in running water and 5 ml of 50% aqueous ethanol was added to each tube. Color development was read as absorbance at 570 mμ on a Bausch and Lomb Spectronic 20 Spectophotometer. Proline was read at 440 mμ. Concentrations of amino acids were calculated using the absorbance method (11).

The acidic amino acids from freeze-dried nematodes were separated on a column of Bio-Rad Aminex-4 as recommended (2) except that the buffer was changed at one hour and fifteen minutes instead of one hr. Basic amino acids were separated on Bio-Rad Aminex-5 resin. Eluates from both columns were processed as described in the preceding paragraph.

RESULTS

The total number of amino acids separated from *H. glycines* extracts was 23 (Tables 1–4). As previously reported (1) only five

TABLE 2. Amino acids in incubation fluids and extracts from adult female *H. glycines* separated on a column of Bio-Rad MSQ 50 Resin.

Amino Acid	3-wk sample[a]		4-wk sample[a]	
	Incubate	Extract	Incubate	Extract
Alanine	26.0[b]	16.0	35.0	17.0
Aspartic Acid	7.5	1.1	13.0	3.8
γ-Amino Butyric Acid	0.1	0.1	0.2	0.8
Glutamic Acid	21.0	28.0	22.0	19.0
Glycine	8.1	3.3	–	3.4
Isoleucine	1.7	1.9	2.3	1.8
Leucine	3.5	3.7	3.6	2.7
Methionine	3.0	2.7	–	2.1
Phenylalanine-Tyrosine	2.4	3.6	0.1	2.7
Proline	7.1	1.6	5.8	17.0
Serine + Asparagine + Glutamine	14.0	35.0	13.0	28.0
Threonine	3.0	–	–	1.7
Valine	2.5	2.4	1.6	2.3

[a] Samples were taken three and four weeks after plants were inoculated.
[b] Each figure is a percentage figure based on the total μM of amino acid/mg of nematode used.

TABLE 3. Total amino acid complement of *H. glycine* females taken at selected intervals following host inoculation.

Amino Acids[a]	19 days	26 days	33 days	40 days
Alanine	12.0	20.0	18.5	16.5
Aspartic Acid	4.9	5.6	3.8	4.1
Cystathionine	2.6	1.3	1.3	0.9
Glutamic Acid	15.5	12.0	11.3	11.0
Glycine	6.3	3.6	3.1	3.2
Isoleucine	2.1	1.1	0.9	1.5
Leucine	4.2	3.1	2.0	1.4
Methionine	4.2	0.9	1.2	1.1
Phenylaine + Tyrosine	–	–	2.0[b]	–
Proline	4.1[b]	16.0	16.5	21.5
Serine + Asparagine + Glutamine	28.0	35.0	28.0	23.0
Threonine	1.7[b]	–	2.0[b]	2.5
Unknown A	–	–	4.1[b]	3.8
Valine	4.1	2.3	2.0	1.7
Arginine	8.4[b]	–[c]	5.7[b]	5.1[b]
Histidine	5.4[b]	–	2.4[b]	4.2[b]
Lysine + Ornithine	14.0[b]	–	7.1[b]	6.8[b]

[a] Amino acids extracted from thoroughly ground nematodes. Separation was on columns of Bio-Rad Aminex 4 and Aminex 5 resins. Data are in terms of % of total μM/of acids/mg nematode tissue.
[b] Only one sample represented. Other figures represent average of two samples.
[c] Aminex 5 column not run.

amino acids were found in larval incubation fluids (Table 1), whereas 18 were detected in adult incubation fluids. Extracts from larvae which had first been incubated yielded 14 amino acids, whereas 21 were extracted from adults which had been incubated.

Alanine, glutamic acid and serine-glutamine-asparagine made up 61–70% of the amino acid total in the incubation solutions (Table 2), and aspartic acid, proline, and glycine comprised another 19–23% of the total. Alanine, glutamic acid and serine-glutamine-asparagine also comprised a larger percentage of amino acids from the extracted nematodes. Proline was the only other major component of the four week extract (Table 2).

Phenylalanine and tyrosine were present on paper chromatograms and from the MSQ 50 column but were detected only once from the Aminex-4 column. The tubes in which they might be expected to appear often contained a slight amount of color, but not enough to be meaningful. Hydroxyproline

TABLE 4. Amino acid complement of *H. glycines* adult females at selected intervals following host inoculation.

	26 days[b]	33 days		40 days	
Amino Acids[a]	Crushed	Crushed	Residue	Crushed	Residue
Alanine	17.0	12.0	11.0	12.0	11.5
Aspartic Acid	2.8	3.0	4.1	2.6	3.5
Cystathionine	1.3	0.7	0.9	0.9	0.9
Glutamic Acid	12.0	15.5	15.5	14.0	12.0
Glycine	3.7	2.6	4.3	3.2	4.6
Isoleucine	1.1	0.5	1.5[b]	0.6	1.4[b]
Leucine	1.7	1.1	2.1	0.6	2.3
Methionine	1.3	1.7	1.7	0.7	2.4[b]
Proline	9.4	10.2	9.2	13.0	14.0
Serine + Glutamine + Asparagine	21.0	26.5	26.0	27.5	22.6
Threonine	1.6	1.9[b]	1.8	1.8	1.7[b]
Unknown A	2.6	4.6[b]	3.7	4.8[b]	4.8[b]
Valine	2.5	1.2	1.6	1.0	1.6
Arginine	7.3	7.5	5.8	5.6	6.4
Histidine	5.3	5.0	3.1	4.9	5.9
Lysine + Ornithine	9.0	9.1	9.0	9.3	13.0

[a] Extracts obtained by gentle crushing of adult females; most eggs and larvae inside remained intact. Data are in terms of % of total μM of amino acids/mg nematode tissue. Separation was on columns of Bio-Rad Aminex 4 and Aminex 5.
[b] Only one sample represented. Other figures are averages of two samples.

and methionine sulfoxide were separated on paper (Table 1) but were not detected in column analyses (Tables 2–4). One peak, unknown A, which behaved as sarcosine on the Aminex-4 resin, had no corresponding spot on the paper chromatograms. One spot on paper corresponded with cystathionine and a column peak corresponded with cystathionine and Djenkolic acid. Based on the color developed with ninhydrin it was concluded to be cystathionine.

Serine, glutamine and asparagine were eluted from the column as one peak (Tables 2–4) and lysine and ornithine were eluted as one peak (Tables 3–4). Based on the spot size on paper chromatograms, serine and glutamine were both in higher concentration than asparagine and the peak was calculated as serine.

Some sand particles adhered to the nematodes and their actual weight was probably less than that measured. As a result, the μ-moles/mg of amino acids of nematodes is probably low. The variability in measured weight probably contributed to the wide variability of amino acid content in each sample. Therefore, the results are given as a percent composition.

Discussion

In the incubation studies five amino acids were given off by larvae. These five were also given off in largest quantities by adults. Alanine and glutamic acid were given off in largest relative amounts. The relative amounts of (i) aspartic acid and alanine in the incubation fluids at three and four weeks; (ii) glycine at three weeks; and (iii) proline at four weeks were larger than the relative amounts extracted from the incubated nematodes. These amino acids appeared to be actively secreted or excreted. The MSQ 50 resin did not resolve these amino acids as well as Aminex-4.

Cystathionine has not been previously reported from nematodes. It is an intermediate in the conversion of methionine to cysteine. Cysteine was not detected but this may have been due to sample size or techniques.

Other workers have indicated that proline may be incorporated in high amounts into egg shells. Clark *et al.* (3) found that proline comprised 38.3% of the amino acids of 24-hr hydrolysates from *Heterodera rostochiensis* egg shells. In *Parascaris* oocytes (4) there are granules which are mostly proteins containing more than 20% proline. These proteins are reported to become separated from the cytoplasm and become part of the egg shell. In the present study, the greatest change in the relative amount of any amino acid was that of proline. There was a significant increase between the 19 day sample and 26 day sample. This coincides with egg production in *H. glycines* which begins at about 17 days and probably reaches a peak at 20–28 days under optimum conditions.

Alanine comprised about 25–35% of the amino acids in incubation fluids and 10–20% of those extracted from the nematodes. In contrast, Smith and Ellenby (10) reported that alanine comprises only about 5% of the free amino acids extracted from white females of *H. rostochiensis.*

The presence of methionine in column analysis indicates that the methionine sulfoxide detected on paper is probably an oxidation product of methionine since no precautions were taken to prevent oxidation of methionine during the preparations of the material for paper chromatography. Probably unknown A and threonine were not demonstrated consistently in the eluates because these amino acids were occasionally unresolved.

The nematodes for the extraction studies were selected by chronological age. However, the physiological age of the nematodes

may be a better measure to use. Van Gundy *et al.* (12) reported that the physiological aging of starving *Meloidogyne javanica* (Treub) Chitwood, larvae occurs faster at 25–35 C than at 15 C. In the present study, the nematodes at 19 days were white females. At 26 days they were yellow females, and at 33 to 40 days they were somewhat larger, but essentially still yellow females. After 40 days some had turned brown. Smith and Ellenby (10) showed greater changes in the relative amino acid content when nematode age was based on the color of the cyst. This is probably a better indication of the physiological condition than is chronological age.

LITERATURE CITED

1. AIST, SHELIA and R. D. RIGGS. 1967. Amino acids from *Heterodera glycines* larvae. Phytopathology 57:801 (Abstr.).
2. BIO-RAD LABORATORIES. 1966. Aminex resins, buffers, reagents for amino acid analyzers. Bio-Rad Bull. 115A4.
3. CLARK, A. J., PATRICIA M. COX and A. M. SHEPHERD. 1967. The chemical composition of the egg shells of the potato-cyst nematode, *Heterodera rostochiensis* Woll. Biochem. J. 104:1056–1060.
4. FAIRBAIRN, D. 1960. The physiology and biochemistry of nematodes. p. 267–269. *In* Sasser, J. N. and W. R. Jenkins (eds.) Nematology. Univ. of North Carolina Press, Chapel Hill, North Carolina.
5. GRABLE, C. I., J. E. PRESLEY, and G. E. TEMPLETON. 1964. Identification of ninhydrin positive components in ethanolic extracts in rice panicles by paper chromatography. Ark. Acad. Sci. Proc. XVIII:31–40.
6. MYERS, R. F. and L. R. KRUSBERG. 1965. Organic substances discharged by plant parasitic nematodes. Phytopathology 55: 429–437.
7. PLAISTED, P. H. 1958. Clearing free amino acid solutions of plant extracts for paper chromatography. Contrib. Boyce Thompson Inst. Plant Res. 19:(3)231–244.
8. RIGGS, R. D. and M. L. HAMBLEN. 1962. Soybean-cyst nematode hosts studies in the family Leguminosae. Ark. Agr. Expt. Sta. Rep. Ser. 110:1–18.
9. ROSS, J. P. and C. A. BRIM. 1957. Resistance of soybeans to the soybean cyst nematode as determined by a double-row method. Pl. Dis. Rep. 41:923–924.
10. SMITH, L. and C. ELLENBY. 1967. Some biochemical changes in the contents of the cyst of *Heterodera rostochiensis* during its maturation. Nematologica 13:395–405.
11. SPACKMAN, D. H. 1962. Instruction manual AIM2, Model 120B amino-acid analyzer. Spinco Division, Beckman Instruments, Inc., Stanford Industrial Park, Palo Alto, Calif.
12. VAN GUNDY, S. D., A. F. BIRD and H. R. WALLACE. 1967. Aging and starvation in larvae of *Meloidogyne javanica* and *Tylenchulus semipenetrans*. Phytopathology 57: 559–571.