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Aminopeptidase-like activities in *Caenorhabditis elegans* and the soybean cyst nematode, *Heterodera glycines*

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Abstract

Aminopeptidase-like activities in crude whole body extracts of the free-living nematode *Caenorhabditis elegans* and the plant parasitic soybean cyst nematode *Heterodera glycines* were examined. General characteristics including pH optima, heat lability, and inactivation of enzyme by organic solvent were the same for the two species. All developmental stages of *H. glycines* exhibited activity. In older females, activity was present primarily in the eggs. Affinity for the substrate L-alanine-4-nitroanilide was the same regardless of the stage examined, and was similar for the two species ($K_m = 2.3 \pm 0.3$ mM for *C. elegans* and 2.9 ± 0.2 mM for *H. glycines*). Nearly all (>95%) of *C. elegans* aminopeptidase-like activity was present in the soluble fraction of the extract, while *H. glycines* activity was distributed between the soluble and membrane fractions. Specific activities of the soluble enzymes were highest in *C. elegans* and *H. glycines* juveniles. The *C. elegans* enzyme was susceptible to a number of aminopeptidase inhibitors, particularly to amastatin and leuhistin, each of which inhibited aminopeptidase-like activity more than 90% at 90 μM. In *H. glycines*, aminopeptidase-like activity was inhibited 39% by amastatin at 900 μM. The apparent molecular weight of the soluble *C. elegans* enzyme is 70–80 kDa. Some activity in *H. glycines* is present in the 70–80 kDa range, but most activity (80–90%) is associated with a very high molecular weight (>240 kDa) component.

Introduction

Nematodes, like other eukaryotes, depend upon proteolytic enzymes for a variety of essential cellular and developmental events, from the processing of newly synthesized proteins and activation of proenzymes and prohormones, to steady state maintenance and clearance of metabolic products (Sarkis *et al.*, 1988; Lilley *et al.*, 1996). Proteolytic enzymes characterized from free-living nematodes have been associated with feeding (Sarkis *et al.*, 1988; Larminie & Johnstone, 1996) and neuropeptide metabolism (Baset *et al.*, 1998). In parasitic species, they serve additional functions essential to mechanisms of infection including the invasion and destruction of host tissues (Larminie & Johnstone, 1996; Tort *et al.*, 1999) and the penetration of the vascular system to gain access to nutrients (Hotez *et al.*, 1990; Pratt *et al.*, 1990; Knox *et al.*, 1993; Rhoads & Fetterer, 1997). Proteases are associated with ecdysis and moulting in animal parasitic nematodes (Gamble *et al.*, 1989; Hotez *et al.*, 1990; Rhoads & Fetterer,

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1998), and in plant parasitic nematodes are often targeted in the search for novel parasite control approaches (Atkinson *et al.*, 1995, 1998). A trypsin-like serine endoprotease was prepared from the plant parasitic root knot nematode *Meloidogyne incognita* (Dasgupta & Ganguly, 1975). Cysteine endoproteases have been detected in the potato cyst nematode *Globodera pallida* (Koritsas & Atkinson, 1994), in the soybean cyst nematode *Heterodera glycines* (Lilley *et al.*, 1996; Urwin *et al.*, 1997), and a gene coding for a serine protease was described in *H. glycines* (Lilley *et al.*, 1997). Aminopeptidases, including those reported in nematodes, are represented by a broad variety of soluble and membrane-bound molecules, from single polypeptides to large multi-subunit complexes (Taylor, 1993; Tort *et al.*, 1999). Recombinant aminopeptidase has been described in *Caenorhabditis elegans* (Baset *et al.*, 1998) and the gene for a microsomal aminopeptidase was cloned in the filariid *Acanthocheilonema vitae* (Harnett *et al.*, 1999). Secreted and membrane-associated aminopeptidase activities have been examined in *Ascaris suum* (Sajid *et al.*, 1997; Rhoads & Fetterer, 1998). We describe soluble and membrane-associated aminopeptidase-like activities prepared from juvenile and adult *H. glycines*, the major pathogen of soybeans in the United States (Kim *et al.*, 1998). Activities are compared with those from *C. elegans*, and the significance of similarities and differences between the enzymes is discussed.

Materials and methods

Heterodera glycines were obtained from sterile root culture (Masler *et al.*, 1999) and large-scale laboratory cultures (Sardanelli & Kenworthy, 1997). Females were collected, sorted according to colour stages of maturation (Young, 1992), and stored at −15°C. Eggs were harvested from freshly collected females by extrusion into water, cleaned of debris by rinsing with water, then stored at −15°C. Juveniles were hatched from eggs in water over a 48 h period, collected by low speed ($800 \times g$) centrifugation, and stored at −15°C. *Caenorhabditis elegans* were reared in liquid culture at 22°C (Chitwood *et al.*, 1995). Worms were washed repeatedly to remove culture medium prior to extraction.

Tissue was processed in water (typically 1:10–1:20 v:v) by disruption with a Polytron homogenizer (Brinkman Instruments, Westbury, New York). After centrifugation ($48\,000 \times g$, 30 min, 4°C) the supernatant was collected, and the pellet washed by disruption and centrifugation as above. This was repeated once. Supernatants were pooled (soluble fraction), dried in the Speed Vac, and stored at −15°C. The washed pellet (membranes) was weighed, and suspended in assay buffer (100 mM Tris, pH 8.1) for further analysis. Total protein was estimated using the microBCA assay (Pierce Chemical Co., Rockford, Illinois) following the manufacturer's instructions.

Soluble enzyme was assayed at either room temperature (26°C) or 37°C in 96-well polystyrene microtitre plates (Corning Easy Wash Assay Plate; Corning, Inc., Corning, New York). Samples in 150 μl assay buffer were combined with 30 μl of substrate, L-alanine-4-nitroanilide hydrochloride (L-Ala-NA; Fluka Chemical Co., Ronkonkoma, New York), with final substrate concentrations of 0.7 to 8 mM. Absorbance was monitored using a microplate reader (Biotek Instruments, Winooski, Vermont). The reaction product, para-nitroaniline (pNA) absorbs 25.7 mAU $nmol^{-1}$ at 410 nm. Activity is expressed as nmol pNA produced per minute at 4 mM L-Ala-NA unless otherwise noted. V_{max} and K_m were estimated using the Lineweaver-Burk equation.

For the assay of enzyme activity in membrane preparations, the membranes were suspended in assay buffer, and the suspension divided among several (at least four) tubes at 210 μl per tube. Substrate (40 μl; final concentrations 0.7 to 8 mM) was added to each tube and the reactions incubated. At 25–30 min intervals, tubes were centrifuged (2 min, $9800 \times g$), and supernatants (200 μl) were transferred to the microtitre plate and absorbance measured. The supernatants were returned to the respective tubes and incubations continued. This sampling was repeated four to five times to generate reaction curves.

Protease inhibitors tested were, aminopeptidase-specific: amastatin, arphaminine B, bestatin, epiamastatin, epibestatin, leuhistin; serine protease-specific: phenylmethyl-sulfonylfluoride (PMSF) and TLCK; cysteine protease-specific: CBZ and E-64 (Calbiochem, La Jolla, California and Sigma Chemical, St Louis, Missouri).

Molecular weights were estimated by high-performance size exclusion chromatography (HP-SEC; Progel 3000, 300 × 7.8 mm with Progel 3000 guard column, 75 × 7.8 mm, Phenomenex, Torrance, California; elution buffer 100 mM Tris, pH 7.0, 300 mM NaCl; 0.5 ml min^{-1} flow). Samples were applied in elution buffer and fractions assayed directly for enzyme activity. The column was calibrated with Dextran blue and a series of protein markers ranging from 18 kDa to 480 kDa (Pierce and Sigma). Additional molecular weight estimates were made using non-denaturing polyacrylamide gel electrophoresis. Soluble extract fractions were prepared in sample buffer (100 mM Tris-glycine, 10% glycerol, 0.0025% bromophenol blue, pH 8.7; Novex/Invitrogen, Carlsbad, California), and fractionated in 4–20% gradient gels with running buffer of 25 mM Tris, 192 mM glycine, pH 8.4 (Novex/Invitrogen). Lanes containing sample were sliced at 5 mM intervals and the slices incubated overnight with 12 mM L-Ala-NA in 500 μl of 100 mM TRIS, pH 8.1. Supernatants were collected and aliquots (300 μl) assayed for absorbance at 410 nm. Distribution of aminopeptidase activity per gel slice was estimated as percent of total absorbance for all slices. Reference markers used were pepsin (34 kDa), bovine serum albumin (66 and 132 kDa), urease (240 and 480 kDa) and ferritin (440 kDa) (Sigma), detected by staining with GelCode (Pierce).

Results

Aminopeptidase-like activity was detected in *C. elegans* and various stages of *H. glycines* using the chromogenic amide substrate L-alanine-4-nitroanilide. For the first time in each of these species, aminopeptidase-like activity was found in both the soluble (supernatant) and membrane (pellet) fractions. The activities were evaluated and partially characterized separately for each of the tissue fractions.

Table 1. Levels of aminopeptidase-like activity in extracts of *Caenorhabditis elegans* and selected stages of *Heterodera glycines*.

Source	Specific activity[1]	Activity per individual[2]
C. elegans	21.63 ± 4.59[a]	0.74 ± 0.16[a]
H. glycines		
females (all)	1.36 ± 0.28[b]	–
(white/yellow)	1.27 ± 0.37[b]	4.72 ± 0.89[b]
(brown)	1.53 ± 0.99[b]	1.42 ± 0.15[c]
juveniles	4.12 ± 1.71[c]	$9.80 \pm 4.30 \times 10^{-3}$[d]
eggs[3]	0.98 ± 0.14[b]	$2.94 \pm 0.81 \times 10^{-3}$[d]

[1] Specific activity is expressed as mean ± sem nMol pNA per min per μg protein. L-Ala NA was 4 mM. Values followed by the same letters are not significantly different ($P > 0.05$).
[2] Activity is expressed as mean ± sem fMol pNA per min per nematode or egg equivalent. L-Ala NA was 4 mM. Values followed by the same letters are not significantly different ($P > 0.05$).
[3] Eggs were collected from all stages.

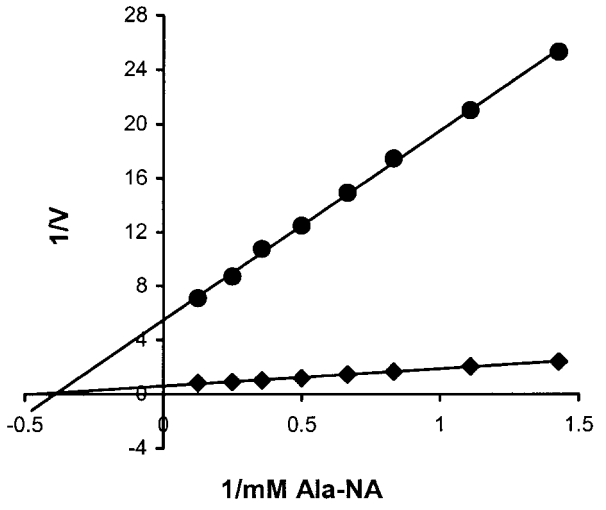

Fig. 1. Typical aminopeptidase activities present in soluble fractions of extracts prepared from *Caenorhabditis elegans* (◆) and *Heterodera glycines* juveniles (●). V=nMol pNA per minute.

A number of basic characteristics of the soluble activity were similar for both *C. elegans* and *H. glycines*. The enzymes from each species were stable to freezing, drying under vacuum and storage (dry) for at least one month. The optimum pH range was 7.8 to 8.3 under the substrate conditions used, with 70–80% of maximum activity retained at a low pH of 6.9 and a high pH of 8.6. Activity increased 1.7 to 2.2-fold when incubation temperature was increased from room temperature to 37°C. Heating at 56°C for 5 min eliminated essentially all activity from preparations of each species. Activity was also destroyed by exposure to 50% acetonitrile in 0.1% trifluoroacetic acid or to 2% SDS, but was not affected by treatment with either DNAse or acetone. The K_m values of soluble aminopeptidase from *H. glycines* (2.9 ± 0.2 mM) and *C. elegans* (2.3 ± 0.3 mM) were not significantly different ($P > 0.05$).

The specific activity of soluble *C. elegans* aminopeptidase was consistently greater ($P < 0.05$) than that from any individual stage of *H. glycines* (table 1). *Caenorhabditis elegans* specific activity was 5-fold greater than that of juveniles, which had the highest specific activity of any of the extracts tested from *H. glycines*. In *H. glycines*, gravid females first appear white, gradually turn yellow and then become brown as they grow and age (Young, 1992), as juveniles develop within the eggs, and as some female protein is lost. During these developmental changes, specific activity in females remains unchanged (table 1). Soluble activity in *C. elegans* was greater per animal ($P < 0.05$) than in *H. glycines* juveniles (table 1). In extracts assayed at the same protein concentration for each species (100 ng μl^{-1}), V_{max} for *C. elegans* aminopeptidase-like activity was 3.7-fold greater than that for *H. glycines* juveniles, but K_m values were the same (fig. 1, results of a typical experiment). The level of soluble activity per *H. glycines* female equivalent was higher than that measured from *C. elegans* or any other *H. glycines* source (table 1). Unlike specific activity, aminopeptidase-like activity per brown female declined more than 3-fold relative to white-yellow females (table 1). Eggs from brown females contain more aminopeptidase-like activity ($5.3 \pm 1.6 \times 10^{-3}$ fMol pNA per min per egg) than do eggs obtained from white and yellow females ($1.8 \pm 0.6 \times 10^{-3}$ fMol pNA per min per egg). Since *H. glycines* females can each have between 100–200 eggs or more, the eggs may account for most of the aminopeptidase-like activity present in brown females. In fact, in duplicate individual assays of whole brown females and cohorts from which eggs and bodies were separated, an average of 62% of the level of activity found in whole females was present in the eggs and 32% of whole female activity was present in the bodies from which the eggs were removed. As *H. glycines* females age, the level of aminopeptidase-like activity in whole animals declines, but the level of activity in the eggs increases.

Responses to protease inhibitors differed between the two species. A series of aminopeptidase inhibitors tested at 90 μM showed varying effects upon *C. elegans* aminopeptidase activity. None was effective against *H. glycines* extracts at this concentration, but 900 μM amastatin inhibited 39% of *H. glycines* aminopeptidase activity. The serine protease inhibitor PMSF was not effective at 90 μM in either *C. elegans* or *H. glycines* extracts, but did inhibit *H. glycines* activity 36% at 200 μM (table 2). No other endopeptidase inhibitors tested were effective against *H. glycines* activity.

Aminopeptidase from *C. elegans* eluted as a single peak of activity from the size exclusion column in a volume between 8.6 and 8.8 ml corresponding to a molecular weight estimate of 70–80 kDa. The K_m determined for this active fraction was identical to the K_m of the nematode extract (column input), and recovery of activity in the 70–80 kDa range was 70% of the input. Aminopeptidase from *H. glycines* juveniles, eluting between 8.6 and 8.8 ml, also appeared as a single peak but accounted for less than 10% of the input activity. The bulk of the activity present in *H. glycines* samples applied to the column was recovered in a fraction eluting between the Dextran and catalase (240 kDa) markers. This suggests activity associated with a very large component of the soluble extract. Similar observations were made using native PAGE analysis. Over 80% of *C. elegans* aminopeptidase-like activity was recovered from

Table 2. Inhibition of aminopeptidase activity in extracts of *Heterodera glycines* and *Caenorhabditis elegans*.

Class	Inhibitor[1]	Percent inhibition	
		C. elegans	*H. glycines*
Aminopeptidase	amastatin	95	0
	amastatin (900 μM)	–	39
	epiamastatin	84	0
	bestatin	17	0
	epibestatin	15	0
	arphaminine B	77	0
	leuhistin	95	0
Serine	PMSF	0	0
	PMSF (200 μM)	4	36
	TLCK (200 μM)	–	0
Cysteine	CBZ (200 μM)	–	0
	E-64 (200 μM)	–	0

[1] Inhibitors were 90 μM except where indicated.

the gel at a location between the bovine serum albumin monomeric (66 kDa) and dimeric (132 kDa) markers. In juvenile and female *H. glycines* preparations, only 5–8% of the total recovered activity was present in this region, and 80–90% of recovered activity was located near the sample origin, compared with 5% of the *C. elegans* total. Ferritin (440 kDa) and urease tetramer (480 kDa) each migrated further into the gel than the large *H. glycines* aminopeptidase component.

Aminopeptidase-like activity was present in membrane fractions from all species and stages examined. As with the enzyme from the soluble fractions, activity doubled from 26°C to 37°C and was lost after heating (56°C, 5 min). K_m values of 4.3 ± 0.3 mM for *C. elegans*, 3.2 ± 0.2 mM for *H. glycines* (average for all stages) and 2.5 ± 0.2 mM for *H. glycines* juveniles were similar to those of the soluble fractions. Amastatin inhibited 93% of *C. elegans* activity at 90 μM but had no effect on *H. glycines* activity at 900 μM. Further examination of enzyme preparations revealed that the distribution of aminopeptidase activity in whole extracts differed significantly ($P < 0.05$) between species. In *C. elegans*, membrane fractions accounted for approximately 5% of the total activity recovered with all remaining activity present in the soluble fraction. More than 50% of total *H. glycines* aminopeptidase-like activity was always present in the membrane fraction regardless of the source. The percent of total aminopeptidase-like activity in membranes varied from 53% in the juveniles to 75% in eggs. The remainder was in the soluble fraction. Incubation of *H. glycines* membranes with 300 mM NaCl in 50 mM Tris-HCl buffer, pH 7.8, at room temperature, solubilized about 30% of membrane activity during the first hour, and a further 5–10% during several additional hours. Ultracentrifugation of the soluble fraction ($100\,000 \times g$; 1 h; 5°C) resulted in a less than 5% loss of soluble activity.

Discussion

Despite differences in morphology, sex and age among the life cycle stages of *H. glycines* examined, all appear to have the same aminopeptidase. In contrast, *H. glycines* and *C. elegans* aminopeptidases differ in the areas of tissue distribution and inhibitor response while sharing some fundamental properties. Comparison of K_m values for all preparations of *H. glycines* with those from *C. elegans* suggests that similar aminopeptidase-like enzymes are present in both species, and each species has aminopeptidase activity associated with protein in the 70–80 kDa range. Along with these similarities between the parasitic and free-living nematodes, a number of important differences in enzyme properties were observed. Aminopeptidases are present in several tissues and sub-cellular fractions in nematodes (Sajid *et al.*, 1997; Smith *et al.*, 1997; Rhoads & Fetterer, 1998), including intestine, muscle, pharynx, and ovaries. While the nature of the *H. glycines* extracts precludes assignment of aminopeptidase-like activity to specific tissues, results show substantial amounts of both soluble and membrane-associated aminopeptidase activities. In *C. elegans* essentially all activity is detected in the soluble fraction. Gimenez-Pardo *et al.* (1999) reported aminopeptidase in both soluble and insoluble fractions from *C. elegans*, but did not indicate relative amounts. Baset *et al.* (1998) showed that recombinant *C. elegans* aminopeptidase activity (AP-1) was almost entirely in the cytosol. In our preparations of *H. glycines*, as much as 40% of all recovered activity is present in the solubilized fraction, but the majority of activity is always associated with membranes.

Nematode aminopeptidases associated with intestinal (*Haemonchus contortus*, Smith *et al.*, 1997) and muscle (*A. suum*, Kubiak *et al.*, 1996; Sajid *et al.*, 1996, 1997) membranes were found to consist of mixtures of integral membrane proteins and enzymes which could be solubilized under varying extraction conditions of detergent or salt. Our results with *H. glycines* demonstrate that alternative extraction conditions for the *H. glycines* enzyme (e.g. inclusion of salts in the extraction medium) can indeed increase the recovery of soluble aminopeptidase-like activity. Nevertheless, notable contrasts exist between *H. glycines* and *C. elegans* in the distribution of aminopeptidase-like activity between soluble and membrane fractions. In the soluble fraction, essentially all *C. elegans* aminopeptidase-like activity is associated with a single molecular weight, whereas with *H. glycines*, two distinct molecular weight ranges are represented.

The soluble vs. membrane activity observations with *H. glycines* and *C. elegans*, the importance of membrane-associated aminopeptidases in animal parasitic nematodes (Sajid *et al.*, 1996, 1997; Smith *et al.*, 1997), and the presence of recombinant *C. elegans* AP-1 almost exclusively in the cytosol (Baset *et al.*, 1998), raise intriguing questions about enzyme-membrane associations in parasitic and non-parasitic nematodes. In addition, there is the difference in response to aminopeptidase inhibitors between *H. glycines* and *C. elegans*. Although these differences could be artefacts generated by specific assay conditions, as was observed with some *C. elegans* protease assays (Gimenez-Pardo *et al.*, 1999), they may indicate important biochemical differences between parasite and non-parasite. Indeed, preliminary examinations (data not given) of two other nematode species, the

free-living nematode *Panagrellus redivivus* and the plant parasitic root knot nematode *M. incognita* revealed that enzyme distribution in *P. redivivus* was the same as observed in *C. elegans*, and the distribution in *M. incognita* was the same as in *H. glycines*, while K_ms were similar for all species. Amastatin (90 μM) had no effect on activity from either of the *M. incognita* fractions, but did inhibit *P. redivivus* membrane and soluble aminopeptidase activities by 65–75%. Important characteristics of aminopeptidase-like activity appear to differ between plant parasites and free-living nematodes, at least within this initial sampling. The effect of PMSF on apparent aminopeptidase activity is curious since serine proteases including subtilisin, trypsin and chymotrypsin are inactive in our assay (data not given).

Aminopeptidase specific activity in *C. elegans* is 5-fold greater than that in *H. glycines* juveniles. This might be explained by differences in substrate preferences or perhaps by the loss of a necessary co-factor during the preparation of *H. glycines* extracts. In any case, structural differences may exist between the aminopeptidases prepared from the two species. A mammalian aminopeptidase and *C. elegans* AP-1 share sequence homology and substrate preferences, suggesting an evolutionary relationship between these nematode and mammalian aminopeptidases (Baset *et al.*, 1998). If there is an evolutionary relationship between *C. elegans* and *H. glycines* or, more broadly, between free-living and plant parasitic nematodes, the differences observed in aminopeptidase characteristics such as tissue distribution and inhibitor response may be consequences of adaptation.

In addition to the notable reduction in aminopeptidase-like activity in *H. glycines* females as they age, there is a marked shift in the distribution of the activity. Younger (white and yellow) females, whose eggs may contain stages from embryonic through developing juveniles, have less than 10% of total female aminopeptidase-like activity in the eggs (using 150 eggs per female as a typical average). By direct measurement, at least 60% of aminopeptidase-like activity in older (brown) females is in the eggs that contain well-developed juveniles ready to hatch. It is interesting to note that the level of aminopeptidase-like activity in a single brown female is essentially equal to that in 150 hatched (J2) juveniles.

Aminopeptidases in nematodes may have a role in neuropeptide metabolism. A family of neuropeptides, FMRFamides, is involved in nematode neuromuscular regulation (Davis & Stretton, 1995; Shaw, 1996). In *A. suum*, membrane-bound and soluble aminopeptidases metabolize FMRFamide and other peptides (Kubiak *et al.*, 1996; Sajid *et al.*, 1996; Rhoads & Fetterer, 1998). A role for *C. elegans* AP-1 in neuropeptide regulation was suggested (Baset *et al.*, 1998), and preliminary results in our laboratory support this role for nematode aminopeptidase. Such findings may lead to new targets for the design of novel plant parasitic nematode control agents.

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