Journal of Nematology 17(3):270–274. 1985.
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Leucine Aminopeptidase in Eggs of the Soybean Cyst Nematode *Heterodera glycines*[1]

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Abstract: Supernatant from a sonicated macerate of eggs of *Heterodera glycines* hydrolyzed L-leucine β-naphthylamide and L-leucine 7-amido-4-methylcoumarin. Rate of substrate hydrolysis was influenced by pH and increased with the duration of incubation. A Michaelis-Menten constant of 0.15 mM was obtained. Rate of substrate hydrolysis was decreased by freezing egg supernatant for 26 days or heating above 60 C for 5 minutes. When egg supernatant was incubated with six different substrates, L-leucine β-naphthylamide was hydrolyzed most readily and L-valine β-naphthylamide the least readily. The rate of substrate hydrolysis by egg supernatant was not increased by pretreatment of eggs with 3 mM zinc chloride for up to 14 days.

Key words: biochemistry, egg hatching, enzyme, *Glycine max*, soybean, nematode eggs.

The hatching mechanisms in cyst nematodes were reviewed recently (10). Most of our knowledge concerns the role of various physical and ionic stimuli in the hatching of eggs. Changes in eggshell permeability had been implicated in the hatching mechanism; however, Rogers (13) proposed that hatching stimuli elicited enzymatic activity in the eggs of zooparasitic nematodes with subsequent hatch following digestion of the eggshell. Leucine aminopeptidase (LAP) and other enzymes were implicated in the processes of hatching and exsheathment of the ruminant nematode *Haemonchus contortus*, based on the enzymatic activity of the hatching and exsheathment fluids (14). Leucine aminopeptidase is a metalloenzyme and requires zinc as a co-factor for activity.

The cyst nematode *Heterodera glycines* is an economically important parasite of soybean plants, but no studies of enzymatic activity during hatching of its eggs have been reported. The biological activity of zinc as a hatching stimulus for *H. glycines* (4) suggests that LAP may be present in eggs of this nematode. The objective of this study was to examine eggs of *H. glycines* for LAP activity.

Materials and Methods

Heterodera glycines Race 3 was maintained on greenhouse-grown soybean plants as reported previously (15). Cysts were washed from the roots and opened with forceps. Eggs were separated from debris with a 250-μm-pore sieve, concentrated by centrifugation at 250 *g* for 5 minutes, and cleaned by centrifugation in a sucrose gradient (1). Eggs were recovered from the sucrose solution and rinsed with 300 ml reagent-grade water on a 0.45-μm-pore filter (Millipore) before centrifugal concentration. The number of eggs was determined microscopically for three 0.1-ml aliquots. Eggs were macerated manually in a Ten Broeck tissue grinder and sonicated 12 times for 15 seconds in 0.05 M tris(hydroxymethyl)aminomethane hydrochloride buffer (tris) (Sigma Chemical Co., St. Louis, Missouri) at pH 8 in an ice bath, Lubrol (Sigma) was added to a final concentration of 0.1%, and the mixture was agitated in an ice bath for 1 hour. After centrifugation for 5 minutes at 500 *g*, the supernatant was tested for LAP activity. In preliminary experiments we calculated enzyme activity per egg-equivalent, whereas in subsequent trials activity per total egg protein (3) was determined.

Leucine aminopeptidase activity was assayed by colorimetry or fluorimetry. Egg supernatant was incubated with L-leucine 7-amido-4-methylcoumarin (Sigma) as the substrate in the fluorimetric assay as reported previously (9). Enzyme activity of egg supernatant was plotted against 0–0.5-mM concentrations of substrate. Background controls used boiled egg supernatant throughout experimentation. In fluoro-

Received for publication 23 August 1984.

[1] Research was supported in part by the Illinois Soybean Program Operating Board. Part of the research was done at Department of Physiology, Southern Illinois University, Carbondale, IL 62901.

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metric estimation of the Michaelis-Menten constant (K_m) for LAP in egg supernatant (17), substrate (0.01–0.32 mM) was incubated with 4,150 egg-equivalents/ml for this determination.

The effect of pH from 4.6 to 8.6 on substrate hydrolysis by egg supernatant was studied using the organic buffers (8) glycylglycine (pH 8.6), Tris·HCl (pH 8.0), N,N-bis(2-hydroxyethyl)2-aminoethanesulfonic acid (pH = 7.5, 7.1), 2-(N-morpholino)ethanesulfonic acid (pH 6.3, 5.7), and di(tris[hydroxymethyl]aminomethane) sulfate (pH 5.2, 4.6) (Sigma). Buffers were adjusted to the desired pH with 2 M HCl or 2 M NaOH; pH was measured before and after incubation. Substrate at 0.16 mM was used with an egg supernatant containing 1,250 egg-equivalents and a protein concentration of 4 μg/ml. Fluorescence was determined immediately after the addition of egg supernatant and again 1 hour later at 23 C.

Although fluorimetry was more sensitive, colorimetry allowed easier changes of solvent, more stability, and easier manipulation. The colorimetric method was developed by Green et al. (7) and modified by Rogers (11) for analysis of nematode fluids. These procedures were followed with L-leucine β-naphthylamide (Sigma) as the substrate unless indicated otherwise. Background controls used boiled egg supernatant. Optical density was determined versus concentrations of 0–0.09 μM naphthylamine in tris buffer.

In one experiment, egg supernatant was divided to measure the rate of substrate hydrolysis. Seven aliquots from 34,000 egg-equivalents were incubated for 0, 0.5, 1, 2, 4, 8, and 16 hours with substrate.

Supernatant from an additional group of cysts contained 75 μg protein/ml and 16,600 egg-equivalents/ml and was used in four experiments. In investigation of the effect of zinc chloride on LAP activity, 150 μl substrate and 150 μl egg supernatant were incubated with buffered 10^{-10} to 10^{-3} M zinc chloride at a final volume of 0.6 ml. In a study of the stability of enzyme activity, egg supernatant was frozen for 2, 12, 26, 46, 53, and 70 days and then thawed and incubated with substrate for 12 hours at 37 C. Heat stability of enzyme activity was studied by heating samples of egg supernatant for 5 minutes at temperatures of 40–70 C in 5-degree increments before incubation with substrate.

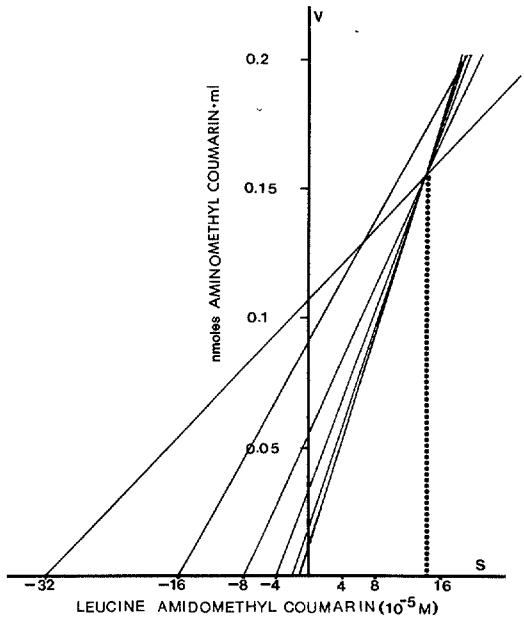

FIG. 1. Linear plot of rates of hydrolysis of L-leucine 7-amido-4-methylcoumarin by preparations of *Heterodera glycines* eggs plotted versus the negative of substrate concentration. Rate of hydrolysis was measured as nmoles aminomethyl coumarin produced by 4,150 egg-equivalents·ml·hr. Intersection of dotted line with X-axis estimated K_m.

In the remaining experiment, the LAP activity in egg supernatant toward various substrates was determined by incubating 150 μl supernatant with L-alanine β-naphthylamide, L-valine β-naphthylamide, L-proline β-naphthylamide, L-isoleucine β-naphthylamide, L-leucine β-naphthylamide, or L-lysine β-naphthylamide (Sigma) in tris-HCl buffer (pH 8.0) in a total volume of 0.6 ml. Substrates were dissolved according to previous procedures (7,11) and added to incubates to make a final concentration of 0.34 mM; reaction mixtures were incubated for 12 hours at 37 C. Enzyme activity was determined in triplicate for each substrate.

Additionally, the effect of zinc pretreatment of eggs on the activity of LAP in supernatant from treated eggs was investigated with another group of cysts. Two groups of 30,000 eggs were used. One group was incubated in 3 mM zinc chloride for 0, 0.5, 1, 3, 5, 7, 10, and 14 days while

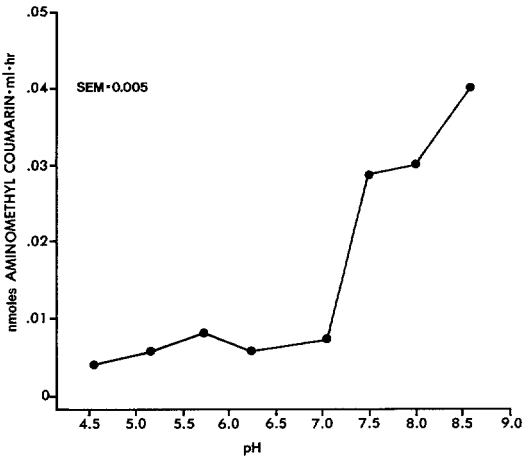

FIG. 2. Aminomethyl coumarin liberated from L-leucine 7-amido-4-methylcoumarin by 1,250 egg-equivalents/ml (4 μg protein/ml) of *Heterodera glycines* preparation at various pH values.

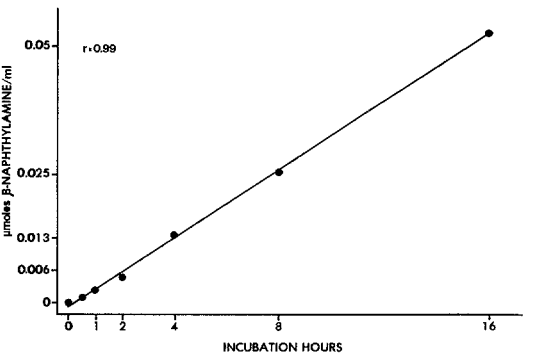

FIG. 3. β-Naphthylamine liberated from L-leucine β-naphthylamide by leucine aminopeptidase in preparations of 34,000 egg-equivalents/ml of *Heterodera glycines* at various incubation times.

another portion was incubated in reagent-grade water rather than tris buffer. The eggs were then rinsed with three changes of 30-ml aliquots of water on a 0.45-μm-pore filter. A small subsample was removed to determine the number of eggs in each sample; the eggs were dispensed with dilution into a microtiter plate and both eggs and juveniles were counted five times over a 2-week period to determine hatch rate. The remaining eggs were rinsed from the filter and sonicated in 1 ml water to measure LAP activity in the egg supernatant. Total protein and egg-equivalents were determined for each sample of egg supernatant.

Data were analyzed by linear regression and analysis of variance. The 0.05 probability level was considered the significance level.

RESULTS

Rates of hydrolysis of various concentrations of L-leucine 7-amido-4-methylcoumarin up to 0.32 mM by LAP in egg supernatant as determined by fluorimetry were concentration-dependent up to 0.08 mM and concentration-independent above 0.16 mM (Fig. 1). An estimate of K_m was determined by drawing a line through the intersection of plotted lines to the X-axis (17). Estimates of the K_m for LAP from egg supernatant of *H. glycines* were 0.15 mM.

Hydrolysis of substrate by LAP from egg supernatant of *H. glycines* increased significantly from pH 7.1 to 8.6 (Fig. 2). Little hydrolysis occurred below pH 7; therefore, subsequent studies were conducted with the reaction mixture of pH 8.

Incubation of egg supernatant (17,000 egg-equivalents/ml) with L-leucine β-naphthylamide resulted in a linear increase ($r = 0.99$) in the release of β-naphthylamide with time (Fig. 3). An incubation period of 12 hours was used in subsequent experiments.

Zinc chloride from 10^{-4} to 10^{-10} M increased the LAP activity of egg supernatant by a mean of 36% when compared to the control, but the rate of hydrolysis was independent of zinc concentration ($r = 0.03$). Egg supernatant with zinc hydrolyzed 0.043 (± 0.004) nmoles of substrate, whereas nontreated egg supernatant hydrolyzed 0.030 (± 0.0) nmoles of substrate. Egg supernatant with zinc chloride at 10^{-3} inhibited enzyme activity by 20%.

LAP activity in egg supernatant frozen at −5 C for up to 10 weeks hydrolyzed L-leucine β-naphthylamide; however, enzymatic activity was only 57% as active as in the original supernatant after 70 days (Table 1). LAP in egg supernatant exposed to 60 C for 5 minutes was 40% as active toward L-leucine β-naphthylamide as was LAP in nonheated supernatant, whereas exposure to 65 C or higher eliminated enzymatic activity.

When compared to a variety of substrates, LAP in egg supernatant hydrolyzed L-leucine β-naphthylamide and L-lysine β-naphthylamide most rapidly (Table

TABLE 1. Quantity of L-leucine β-naphthylamide hydrolyzed by leucine aminopeptidase in preparations from *Heterodera glycines* eggs after freezing for 0–70 days.

Days frozen at −5 C	nmoles·ml·hour·μg protein
0	0.066
2	0.070
12	0.074
26	0.071
46	0.061
53	0.050
70	0.042
$\bar{x}$	0.062
SEM	0.0045

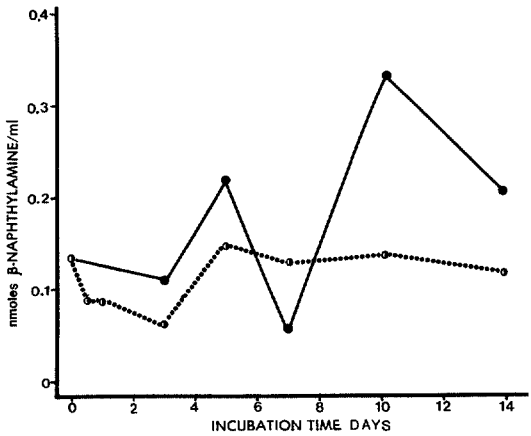

FIG. 4. β-Naphthylamine liberated from L-leucine β-naphthylamide by leucine aminopeptidase/mg protein in preparations from 30,000 egg-equivalents/ml of *Heterodera glycines* treated with water (●) or 3 mM zinc chloride (◑) for 0 to 14 days.

2). Some hydrolysis of the alanine and little hydrolysis of the isoleucine, proline, and valine β-naphthylamides occurred.

Activity of LAP in supernatant of eggs of *H. glycines* exposed to 3 mM zinc chloride for various time periods was not correlated ($r = 0.32$) to exposure time (Fig. 4). Eggs exposed to zinc chloride had a 67% hatch rate after 14 days and egg supernatant contained 10.6 μg protein/ml. Unexposed eggs had a 7% hatch rate and 5.3 μg protein/ml, based on regression analysis after 14 days of incubation. LAP in supernatant from eggs exposed to zinc chloride hydrolyzed less L-leucine β-naphthylamide (mean = 0.12 ± 0.012 nM·ml·hr) than did LAP in supernatant from eggs exposed to water (mean = 0.18 ± 0.043 nM·ml·hr).

DISCUSSION

Zinc may be involved as a cofactor for enzyme activity in the hatching of *H. glycines* eggs. Previous study indicated that hatching of *H. glycines* increases with the duration of zinc exposure and is dependent on pH; in addition, hatching in solutions containing zinc ions at various temperatures reveals a high Q_{10} (3.64) and is inhibited by chelation of the zinc (16). These data suggest that a zinc-dependent enzyme may mediate hatching of *H. glycines* eggs. We report the occurrence of leucine aminopeptidase activity in the egg supernatant of *H. glycines*. The activity of *H. glycines* LAP in crude preparations at various pH values is similar to the activity of mammalian LAP (9). Additionally, the K_m of 0.15 mM is similar to that reported for porcine LAP (0.16 mM) under similar conditions (9).

TABLE 2. Mean rate of hydrolysis of several substrates at a concentration of 0.34 mM by leucine aminopeptidase from preparations of *Heterodera glycines* eggs.

Substrate	nmoles·ml·hour·μg protein (± SEM)
L-leucine β-naphthylamide	0.085 (0.026)
L-lysine β-naphthylamide	0.057 (0.016)
L-alanine β-naphthylamide	0.043 (0.000)
L-proline β-naphthylamide	0.030 (0.003)
L-valine β-naphthylamide	0.025 (0.001)
L-isoleucine β-naphthylamide	0.005 (0.000)

LAP activity has been found in preparations from eggs of at least two other nematode species (14), but any function of this enzyme in hatching is not known for certain since pseudocollagenase, another zinc metalloenzyme, also occurs in eggs of the nematode *Haemonchus contortus* (12). Also, zinc binds to mammalian LAP at two distinct sites, one of which is catalytic and the other regulatory (6). Thus, the zinc-dependent regulatory site may modulate the enzymatic activity of the catalytic site. Anions can also affect enzymatic activity, as mammalian LAP activity in chloride is less than in sulfate (6). Hatching of *H. glycines* eggs in zinc chloride and zinc sulfate is different (16).

Mammalian LAP is not selective for leucine residues; it hydrolyzes any amino acid

with a free α-amino group (2). Our study suggests that LAP in eggs of *H. glycines* preferentially hydrolyzes peptide bonds in the descending order—leucine, lysine, alanine, proline, valine, and isoleucine. Interestingly, the order of prevalence of these amino acids in eggshells of the related cyst nematode *Globodera rostochiensis* is in descending order—proline, lysine, alanine, leucine, valine, and isoleucine (5). Thus, the order of hydrolysis by nematode LAP is unrelated to relative prevalence of an amino acid in the eggshell if one assumes a similar amino acid profile for eggshells of *H. glycines.*

LAP activity in egg supernatant remains, although at a diminished level, after heating at 60 C or freezing at −5 C. Thus, temperate environmental conditions may have only a minor deleterious effect on any LAP activity and related hatching of *H. glycines* eggs.

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