Juvenile Hormone Esterase Activity Repressive Factor in the Plasma of Parasitized Insect Larvae*

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A proteinaceous factor that represses plasma juvenile hormone esterase activity in parasitized insect larvae has been isolated and partially characterized from last instar larvae of the armyworm Pseudaletia separata parasitized with the wasp Apanteles kariyi. Purification procedures consisted of extraction with 25% ethanol, gel filtration and reversed phase high performance liquid chromatography. Plasma juvenile hormone esterase activity in Day 3 last instar larvae was repressed by 50% when larvae were injected on Days 1 and 2 with 6.5 pmol of the purified peptide, which has a molecular weight of about 4500 Da. The application of the factor also causes more than a 2-day delay in the onset of pupation. The sequence of 23 amino acid residues at the amino terminus of the factor was determined as follows: H-Glu-Asn-Phe-Ser-Gly-Gly-Xaa-Val-Ala-Gly-Tyr-Met-Arg-Thr-Pro-Asp-Gly-Xaa-Lys-Pro-Thr-Phe-Tyr-Gln.

Endoparasitic insects disrupt the metamorphosis of holometabolous host insects with the development often arrested in the larval stages (1). The specific mechanisms whereby parasitism causes suppressed metamorphosis of host insects remain enigmatic and little progress has been made toward resolving the question until recently. Last larval stages of the armyworm Pseudaletia separata parasitized with eggs of the parasitoid wasp Apanteles kariyi do not initiate metamorphosis and the wasp larvae emerge from the host larvae about 10 days after parasitization (2). Studies on the endocrinological nature of the effect indicate that the parasitoid inhibits prothoracotropic hormone synthesis and secretion by the host larval brain (3, 4). The role of JH in controlling the release of prothoracotropic hormone has been investigated (3-6) and, in early last instar larvae, the absence of JH from plasma is necessary for release of prothoracotropic hormone from the brain (3). In last instar larvae of most Lepidoptera, the first burst of JH esterase activity correlates with a decline in plasma JH, after which prothoracotropic hormone is released and initiates a cascade of events leading to pupation (7, 8). In the light of this proposed scheme, it was of interest to examine JH esterase activity in the plasma of parasitized larvae. In this study I have demonstrated the existence of a repressive factor that blocks the first peak of plasma JH esterase activity in the parasitized last instar larvae. Isolation and characterization of the factor was also carried out. In addition, the effect of the factor on the metamorphosis of the host larva was also tested.

MATERIALS AND METHODS

Animals—P. separata were reared on an artificial diet at 25 ± 1 °C with a photoperiod of 16 h light/8 h dark (2). Parasitization by A. kariyi was carried out by exposing prospective hosts (usually Day 0 or Day 1 last instar larvae) to female wasps. After a single oviposition was observed the parasitized larvae were quickly removed in order to avoid superparasitization and then reared on the artificial diet. Adult wasps were maintained with honey.

Chemicals—[10-3H]JH I (155 Ci/mmol) and peptide 4-methylcoumarin amide were purchased from DuPont and Peptide institute, Inc., Japan, respectively.

Preparation of Plasma—The hemolymph of parasitized Day 4-6 last instar larvae (3-6 days after parasitization) was collected into a chilled polypropylene test tube containing 500 μl of 0.05% phenylthiourea in 0.2 M phosphate buffer, pH 6.0 by the "flushing out" method with an injection of about 0.1 ml of 0.1 M phosphate buffer containing 100 mM KCl and 10 mM EDTA, pH 6.0, into each hemocoele (9). Collected hemolymph was immediately centrifuged at 4 °C for 5 min at 500 X g and the supernatant was used as the plasma sample for purifying the JH esterase repressive factor. Ten milliliters of plasma was collected from about 80 parasitized last instar larvae.

Enzyme Assay—Ten microliters of the hemolymph was collected from a test larva and incubated in a microcentrifuge tube containing 110 μl of 0.02% phenylthiourea in 0.2 M phosphate buffer, pH 6.0, and 1.5 μmol of substrate (final concentration = 5 X 10-5 M).

Plasma degradation of [10-3H]JH I and unlabeled carrier hormone were measured in vitro using the methods of Hammock and Roe (10). Every enzyme preparation (plasma) was first preincubated with 2 X 10-4 M diisopropyl fluorophosphohosphate (Sigma) at 25 °C for 10 min prior to addition of substrate (final substrate concentration = 5 X 10-5 M).

Plasma without preincubating with diisopropyl fluorophosphohosphate was used as enzyme preparation for assay of peptide 4-methylcoumarin amide (tert-butoxycarbonyl-Val-Pro-Arg-4-methylcoumarin amide) hydrolyzing activity. The release of 7-amino-4-methylcoumarin from peptide 4-methylcoumarin amide during the hydrolysis enzyme reaction was detected using a fluorescence spectrophotometer according to the methods of Morita et al. (11).

Determination of Repressive Factor—The purified factor was diluted with Ringer's solution to the desired concentration (final volume = 10 μl) and injected into unparsitized last instar larvae at Day 1 and Day 2. The next day, plasma was prepared from the larvae for assay of JH esterase activity. Controls were injected with 10 μl of Ringer's solution only. One unit of repressive activity decreased 10% of plasma JH esterase activity compared with the control under the conditions described above.

Protein Determination and Characterization—Protein was determined by the method of Bradford (12) using the Bio-Rad protein assay kit with bovine serum albumin as standard.

The NH2-terminal amino acid sequence of purified JH esterase repressive factor was analyzed by automated Edman degradation with a protein sequencer (model 477A, Applied Biosystems) (13). The sequence was verified by analyzing 100-200 pmol of the native purified factor 5 times.

Samples of purified repressive factor were prepared for SDS-polyacrylamide gel electrophoresis by incubating with 80 mM Tris- HCl buffer, pH 8.8, containing 2% SDS and 2.5% mercaptoethanol in boiling water for 5 min and developed by the methods of Laemmli.
RESULTS AND DISCUSSION

In unparasitized last instar larvae (Day 3 and Day 6) of *P. separata*, two major bursts of plasma JH esterase activity were observed in agreement with reports for other lepidopterous larvae (7). However, no burst of JH esterase activity occurred in parasitized larval plasma during the last larval instar (Fig. 1). A similar observation has been reported in the larval hemolymph of the tobacco hornworm parasitized by the braconid wasp (16). These results suggest the presence of a factor that inhibits JH esterase activity directly or indirectly in the plasma of parasitized larvae. Although preliminary experiments failed to demonstrate an inhibitor functioning directly against JH esterase, the injection of plasma from parasitized last instar larvae into unparasitized larvae at Days 1 and 2 resulted in repression of JH esterase activity in Day 3 larvae. This finding was interpreted to indicate that a JH esterase repressive factor functions in vivo in parasitized last instar larval plasma.

The plasma collected from parasitized last instar larvae was mixed with -20 °C ethanol (final concentration = 25%), and, following centrifugation at 4 °C for 15 min at 20,000 × g, the supernatant was concentrated by lyophilization and applied to a 3-ml disposable C₁₈ extraction column (J. T. Baker Chemical Co.) equilibrated with 6 ml of distilled water. The column was washed with 5 ml of distilled water and subsequently eluted with 2.5 ml of 30% acetonitrile. The eluent was concentrated under a stream of nitrogen and applied to gel permeation chromatography on Superose 6 HR 10/30 (Pharmacia LKB Biotechnology Inc.) equilibrated with 12.5 mM phosphate buffer, pH 6.6. The bioactive zone was collected and further resolved by a reversed phase C₁₈ HPLC column (Yamamura Co., Japan; 4.6 mm inner diameter × 250 mm, pore size = 300 Å) using gradient elution from 18 to 40% CH₃CN in 0.1% CF₃COOH/H₂O at a flow rate of 0.4 ml/min. The bioactive fraction was again collected and rechromatographed using a reversed phase cyanopropyl-derived silica HPLC column (Yamamura Co.; 4.6 mm inner diameter × 250 mm, pore size = 300 Å) under three elution schedules as follows (Fig. 2). The active fraction of C₁₈ was first eluted using a gradient of CH₃CN from 15 to 25% in 0.1% CF₃COOH/H₂O at a flow rate of 0.4 ml/min (Fig. 2A) and then reapplied and eluted with a gradient of 18 to 25% CH₃CN in 0.05% CF₃CN1/C₆CN/H₂O at a flow rate of 0.4 ml/min (Fig. 2B). The fraction with repressive activity eluted in a single peak and was reapplied to the same column and then eluted using a gradient of 20 to 25% CH₃CN in 0.075% CF₃CF₂CF₂COOH/H₂O at a flow rate of 0.4 ml/min (Fig. 2C). Typical data on the purification process of the repressive factor are summarized in Table I.

SDS-polyacrylamide gel electrophoresis of the purified repressive factor yielded a single band that indicates a molecular weight of about 4,500 as shown in Fig. 3. The NH₂-terminal amino acid sequence for the first 23 residues was determined as follows: H-Glu-Asn-Phe-Ser-Gly-Gly-Xaa-Val-Ala-Gly-Tyr-Met-Arg-Thr-Pro-Asp-Gly-Arg-Xaa-Lys-Pro-Thr-Phe-Tyr-Gln-. Sequence comparison was made against the National Biomedical Research Foundation Protein Data Bank using Beckman's MicroGenie version 4.0. Limited sequence similarity was observed with internal sequence of several...
proteins including influenza A virus neuraminidase (EC 3.2.1.18) (67% similarity for 12 amino acids of the repressive factor).

The purified repressive factor shows a dose-dependent capacity to decrease plasma JH esterase activity as shown in Fig. 4. Injection of about 6.5 pmol of the purified factor into unparasitized last instar larvae at Day 1 and Day 2, respectively, reduced plasma JH esterase activity by 50% compared with control (see "Materials and Methods"). In contrast, the other hydrolase activity, peptide 4-methylcoumarin amide hydrolyzing activity, was not changed at all by the injection (Table II). Furthermore, the application of about 7 pmol of the purified repressive factor at Days 1 and 2, respectively, also resulted in more than a 2-day delay in pupation in 80% of the larvae (Table III).

As long as juvenile hormone concentration maintains its high level in insect larval plasma (hemolymph without cells), the insect never undergoes metamorphosis from larva to pupa (8). Since the JH esterase found in larval plasma undoubtedly accounts for the major hydrolysis of JH (7), it can be said that plasma JH esterase is a key enzyme for controlling insect metamorphosis. The factor that I reported here represses plasma JH esterase activity in the last instar larvae and consequently causes a delay in the onset of pupation. The prolonged application of about 6 pmol of the purified repressive factor into the last instar larvae once every day from Day 0 to Day 5 causes more than a 5-day delay in pupation (data not shown). This observation together with the results in Table III strongly suggests that a cascade of events leading to pupation will not initiate as long as the repressive factor exists in the host larvae. It is evident that the repressive factor may perturb the endocrinological processes that control normal metamorphosis in the host insect although the site of action of the factor is still unknown. As a result of the action of the repressive factor, the onset of host pupation is delayed and the parasitoid has adequate time in which to complete growth and development, especially when parasitization occurs during the late stages of larval development of the host.

In summary, this study clearly shows the existence of a JH

| Enzyme activity   | Hydrolyzing JH | Hydrolyzing peptide-MCA* |
|-------------------|---------------|--------------------------|
| Control           | 18.5 ± 3.00   | 218 ± 111               |
| Treated           | 5.72 ± 0.902  | 304 ± 82.5              |

* MCA, peptide 4-methylcoumarin amide.

Each enzyme activity is the mean ± S.D. of four separate assays.
JH Esterase Repressive Factor in Parasitized Insect

TABLE III
Effect of injection of the JH esterase repressive factor on the timing of pupation of unparasitized last instar larvae

| No. of hosts examined | Stage* | Day of last instar larvae |
|----------------------|--------|--------------------------|
|                      |        | 1st 2* 3 4 5 6 7 8 9 10 11 12 |
| Control              |        | L 16 16 16 16 16 16 16 16 16 |
|                      | PP     | 16 16 16 16 16 15 3 11 11 3 |
|                      | P      | 16 16 16 16 16 15 3 11 11 3 |
|                      | D      | 16 16 16 16 16 15 3 11 11 3 |
| Treated              |        | L 17 17 17 17 17 17 17 17 17 17 14 5 1 |
|                      | PP     | 17 17 17 17 17 17 17 17 17 17 17 17 17 4 3 |
|                      | P      | 17 17 17 17 17 17 17 17 17 17 17 17 17 12 15 |
|                      | D      | 17 17 17 17 17 17 17 17 17 17 17 17 17 12 15 |

* L, larva; PP, prepupa; P, pupa; D, dead.
* Ringer solution (for "Control") or 7 pmol of the purified repressive factor (for "Treated") was injected into unparasitized last instar larvae at Day 1 and Day 2.

The esterase activity repressive factor in parasitized larval insects. An overall view of the properties of the factor can also be derived from the data. Although the current study does not explain the mechanism by which the repressive factor decreases plasma JH esterase activity, this is probably the first report concerning a natural factor in insect plasma that is responsible for the interruption of metamorphosis by modulating JH esterase activity.

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