

# Recombinant juvenile hormone esterase, an effective tool for modifying juvenile hormone-dependent expression of the early trypsin gene in mosquitoes

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## Abstract

The study of the changes in the steady-state levels of the early trypsin (ET) messenger RNA (mRNA) was used as a sensitive assay for measuring the effects of recombinant juvenile hormone esterase (rJHE) on juvenile hormone (JH)-dependent gene expression in mosquitoes. ET is a female-specific protease present in the midgut of the yellow fever mosquito *Aedes aegypti* during the first few hours after ingestion of a blood meal. Transcription of the early trypsin gene is part of the normal postemergence maturation of the midgut in the adult female, and it is regulated by JH. JHE was cloned from *Heliothis virescens* and expressed in a baculovirus vector. Injection of rJHE into mosquitoes resulted in an increase of JHE activity in the haemolymph. Injection of rJHE into newly emerged adult females delayed the normal increase in steady-state levels of ET mRNA observed in controls. Topically applied methoprene (a JH analogue) reversed the effect of rJHE. Injection of increasing concentrations of rJHE into 3-day-old unfed females resulted in a dose-dependent decrease in the steady-state levels of ET mRNA after 24 h. The effect of rJHE was transient, once the enzyme was cleared (72 h after injection), the steady-state levels of ET mRNA were restored. The injection of rJHE is an effective tool for modifying JH-dependent expression of the early trypsin gene in mosquitoes.

**Keywords:** mosquito, juvenile hormone esterase, early trypsin, gene expression.

## Introduction

Juvenile hormone (JH) regulates several aspects of reproductive maturation in the adult female mosquito, including previtellogenic oocyte growth and fat body competence (Hagedorn, 1994; Klowden, 1997). The titre of JH is regulated by the rate of biosynthesis in the corpora allata and by the rate of degradation in different tissues. Hydrolytic degradation is primarily effected by two classes of enzymes: juvenile hormone esterase (JHE) and epoxide hydrolase (Hammock, 1985). The use of recombinant JHE (rJHE) as a biochemical anti-JH agent has been established for several insects; all of these studies analyzed the effect of rJHE on physiological parameters such as cuticular blackening in *Manduca sexta* (Philpott & Hammock, 1990) and ovarian development in *Acheta domesticus* (Bonning *et al.*, 1997) and *Aedes aegypti* (Harshman *et al.*, 1991).

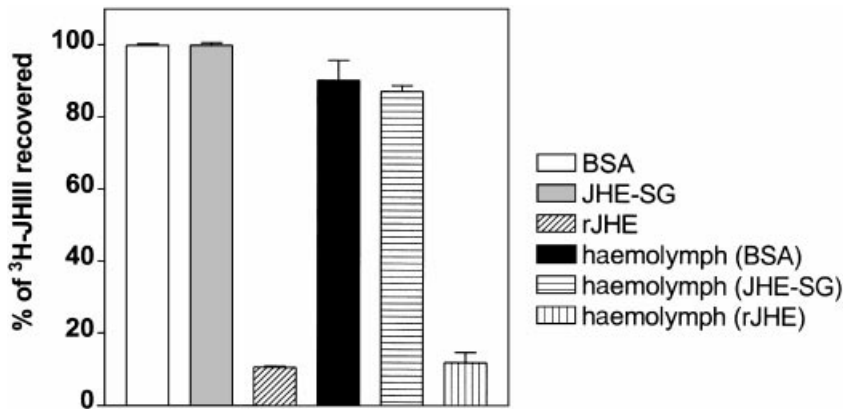
In this paper we describe experiments showing that rJHE is an effective tool for modifying JH-dependent expression of the early trypsin gene in mosquitoes.

## Results

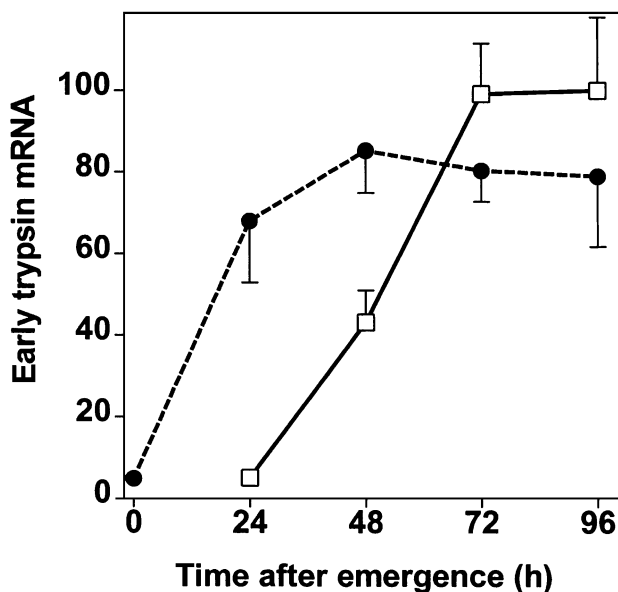
### *Haemolymph from mosquitoes injected with recombinant juvenile hormone esterase-hydrolyzed <sup>3</sup>H-juvenile hormone*

JHE activity was measured using the assay described by Hammock & Sparks (1977). rJHE rapidly hydrolyzed <sup>3</sup>H-JH-III, while bovine serum albumin (BSA) or a recombinant juvenile hormone esterase mutant (JHE-SG) (Ward *et al.*, 1992) did not (Fig. 1). To test if rJHE maintains this enzymatic activity once injected into the mosquito, insects were injected with 250 units of rJHE (where one unit is 40 pmol JH-III hydrolysed per minute), 1 h later haemolymph was collected and the esterase activity was assayed. Controls were injected with identical volumes and protein concentration of BSA or the rJHE mutant (190 ng/0.5 µl)

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**Figure 1.** JHE assay. Assays were performed using pure proteins (190 ng/assay) or haemolymph from insects injected with pure proteins. Three-day-old females were injected with 190 ng of rJHE, JHE-SG or BSA. One hour later, haemolymph from groups of five insects was collected and pooled. Values are the mean  $\pm$  SD of three independent assays. Results are expressed as percentage of intact substrate (<sup>3</sup>H-JH-III) recovered after the assay.



**Figure 2.** Recombinant JHE delayed the expression of the early trypsin gene following adult emergence. Newly emerged females were injected with 250 rJHE units (□) where one unit is 40 pmol JH-III hydrolyzed per minute, or Grace's medium (●). Each point represents the mean  $\pm$  SD of three independent assays of three groups of five mosquitoes. Relative early trypsin mRNA levels are expressed as proportion of the maximum value observed, and are presented as cpm of early trypsin mRNA after background subtraction, multiplied by 100/maximum value (96 h JHE).

(Fig. 1). We observed that rJHE derived from *Heliothis virescens* retains its enzymatic activity in mosquito haemolymph. The majority of active enzyme injected was recovered from injected mosquitoes.

*Recombinant juvenile hormone esterase delayed the expression of the early trypsin gene on newly emerged females*

Insects were injected within 3–4 h after emergence with 250 units of rJHE and the levels of early trypsin (ET) messenger

RNA (mRNA) were measured by Northern blot hybridization. ET mRNA levels are undetectable in newly emerged females, but 24 h after emergence, females injected with Grace's medium have a fortyfold increase in ET transcript levels (identical to non-injected controls). In contrast, injection of rJHE prevented the increase in steady-state levels of ET mRNA observed in controls (Fig. 2). The effect of rJHE was transient; 72 h after emergence the ET mRNA levels in females injected with rJHE increased, and were equivalent to the levels in control insects.

*Topically applied methoprene restored the expression of the early trypsin gene in mosquitoes injected with recombinant juvenile hormone esterase*

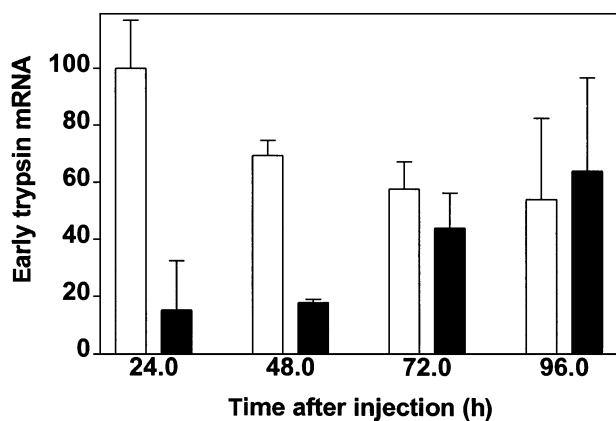
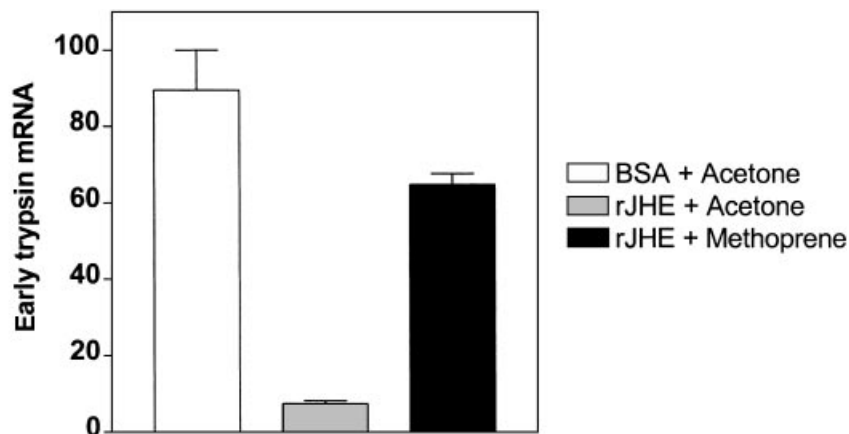
Insects were injected within 3–4 h after emergence with 250 units of rJHE or a similar protein concentration of BSA (190 ng). Methoprene (500 ng/1  $\mu$ l) or 1  $\mu$ l of acetone were topically applied to injected insects. Twenty-four hours after the injection, the levels of ET mRNA were measured by Northern blot hybridization (Fig. 3).

Injection of rJHE prevented the increase in steady-state levels of ET mRNA observed in non-injected insects or in BSA injected controls. Topically applied methoprene induced transcription of the early trypsin gene in rJHE-injected mosquitoes to levels comparable to those observed in control females.

*Injection of recombinant juvenile hormone esterase reduced early trypsin messenger RNA steady-state levels in unfed females*

ET mRNA levels are high in unfed females. Three-day-old adult females were injected with 250 units of JHE and steady-state levels of ET mRNA were measured at different times after injection (Fig. 4). The levels of ET mRNA remained high during the first 12 h after injection of rJHE (results not shown), then a decrease was observed at 24 and 48 h, and by 72 and 96 h, transcript levels had returned to control values (injected with Grace's medium).

**Figure 3.** Effect of methoprene on the expression of early trypsin in mosquitoes injected with rJHE. Newly emerged females were injected with 250 units of rJHE or with BSA (190 ng). Injected insects were topically applied with methoprene (500 ng) or acetone (1  $\mu$ l). Each value represents the mean  $\pm$  SD of three independent assays of three groups of five mosquitoes. Relative early trypsin mRNA levels are expressed as proportion of the maximum value observed, and are presented as cpm of early trypsin mRNA after background subtraction, multiplied by 100/maximum value (BSA + acetone).



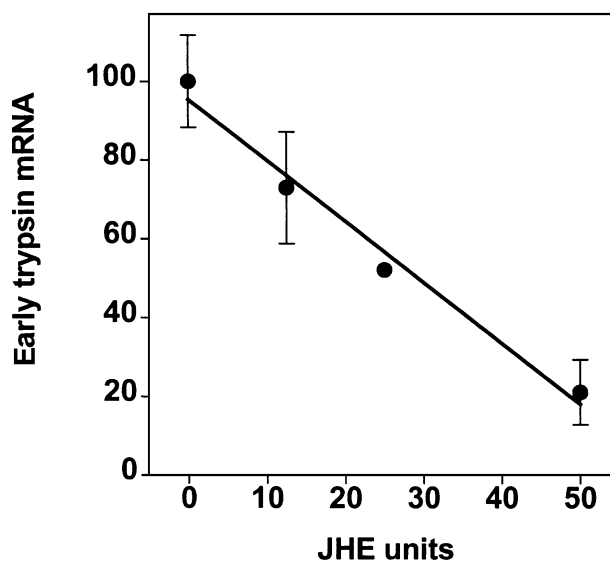
**Figure 4.** Recombinant JHE reduced early trypsin mRNA steady-state levels in unfed females. Three-day-old unfed females were injected with Grace's medium (open bars) or 250 rJHE units (filled bars). Each point represents the mean  $\pm$  SD of three independent assays of three groups of five mosquitoes. Relative early trypsin mRNA levels are expressed as proportion of the maximum value observed, and are presented as cpm of early trypsin mRNA after background subtraction, multiplied by 100/maximum value (24 h Grace's).

*Injection of recombinant juvenile hormone esterase correlated with a dose-dependent decrease of early trypsin messenger RNA steady-state levels in unfed females*

Adult females, 3 days after emergence, were injected with different doses of rJHE diluted in Grace's medium (Fig. 5). The levels of early trypsin mRNA were measured 24 h later by Northern blot hybridization. A dose-dependent effect was evident. Injection of more than 50 JHE units did not result in any further significant reduction in ET mRNA levels.

### Discussion

The usefulness of rJHE as a biochemical anti-JH agent has been demonstrated for several insects (Philpott &



**Figure 5.** Recombinant JHE caused a dose-response decrease of early trypsin mRNA steady-state levels. Adults were injected 3 days after emergence with different doses of rJHE (where one unit is 40 pmol JH-III hydrolyzed per minute). Early trypsin mRNA was measured 24 h later. Each point represents the mean  $\pm$  SD of three independent assays of three groups of five mosquitoes. Relative early trypsin mRNA levels are expressed as proportion of the maximum value observed, and are presented as cpm of early trypsin mRNA after background subtraction, multiplied by 100/maximum value (0 units). The line is a linear regression line ( $r^2 = 0.98$ ).

Hammock, 1990; Bonning *et al.*, 1997). All of these previous studies analysed the effect of JHE on physiological parameters and this is the first report that shows that JH-dependent gene expression can be directly modified using the enzymatic activity of rJHE.

Shapiro *et al.* (1986) showed an apparent inverse correlation between the patterns of JH and JHE levels in adult female *Aedes aegypti*. JH levels were high before feeding and declined after a blood meal, while JHE levels were the opposite. Harshman *et al.* (1991) reported that injection of rJHE into *Aedes aegypti* larvae and pupae

resulted in a dose-dependent decrease in survival and impairment of ovariole maturation.

JH regulates the expression of the ET gene in the *Aedes aegypti* midgut (Noriega *et al.*, 1997). Abdominal ligation within 1 h of emergence, which isolates the midgut from the corpora allata, completely prevented the transcription of the early trypsin gene. Low doses of a JH analogue (methoprene) or higher doses of JH-III restored the expression of the early trypsin gene in ligated abdomens. The study of the changes in the steady-state levels of the ET mRNA was used as a sensitive assay for measuring the effects of rJHE on JH-dependent gene expression in mosquitoes. We observed that injected rJHE was active when injected in the mosquitoes, while injections of other proteins, including an inactive rJHE mutant, JHE-SG, which lacks the active site serine (Ward *et al.*, 1992), did not increase the JHE activity in the haemolymph.

In this study we have shown that rJHE is as effective as abdominal ligation in preventing the expression of the ET gene in newly emerged females. The effect of rJHE is transient with levels of ET mRNA restored to that of controls by 72 h after injection. In two lepidopteran species, clearance of the enzyme from the haemolymph by nephrocytes has been described (Booth *et al.*, 1992; Ichinose *et al.*, 1992); a similar mechanism may be responsible for the transient nature of the rJHE effect in mosquitoes. This transient effect was also observed when 3-day-old females were injected with rJHE. Consequently, even at the time of high JH-biosynthetic activity, rJHE injected into the haemolymph can induce an anti-JH response, indicating that the JH circulating in the haemolymph was metabolized. Additional evidence that rJHE has degraded JH comes from the observation that methoprene reversed the effect of rJHE injection.

rJHE is an excellent biochemical anti-JH agent. Other methodologies such as ligation (Noriega *et al.*, 1997), allatectomy (Raikhel & Lea, 1985) and the use of molecules that affect the biosynthetic activity of the corpora allata (Quistad *et al.*, 1981; Sparks *et al.*, 1987) can result in severe trauma or have other pharmacological effects. On the other hand, by using rJHE the levels of JH can be transiently modulated and depending on the concentration used, the time of injection and the time at which the insects are assayed, different adjustments in the levels of JH, and therefore JH-dependent gene expression, can be observed. rJHE expressed in the mosquito by a suitable expression vector may provide a useful tool for physiological studies and may have application for mosquito control.

## Experimental procedures

### *Insects*

*Aedes aegypti* of the Rockefeller strain were reared at 27°C and

80% relative humidity under a 12 : 12-h light–dark photoperiod regimen. Newly emerged adults were collected every hour from 8:00 to 18:00 hours. Adults were supplied with a cotton-wool pad soaked in a 10% sucrose solution.

### *Recombinant juvenile hormone esterase production*

Recombinant JHE derived from the lepidopteran *H. virescens* was produced by infection of the cell line Tn5B1–4 'High Five' (Invitrogen, La Jolla, CA) in serum-free medium, with the recombinant baculovirus AcUW2(B)JHE (Bonning *et al.*, 1992). rJHE was purified by Q-Sepharose ion exchange chromatography (High Performance Q-Sepharose) (Pharmacia Biotech). Cell culture medium containing rJHE was diluted 1 : 2 with Tris–phosphate buffer pH 8.5 (50 mM Tris, 5% sucrose, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.02% sodium azide). rJHE was eluted from the column by using a sodium chloride gradient (0–200 mM) in Tris–phosphate buffer pH 7.5, and fractions containing JHE were concentrated on a Centricon 30 filter (Amicon, Beverly, MA). Before injection samples were diluted using Grace's medium (Sigma, St Louis, MO).

A recombinant juvenile esterase mutant with a serine at the catalytic site mutated to a glycine residue (AcJHE-SG) was constructed and purified as previously described (Ward *et al.*, 1992; Bonning *et al.*, 1995).

### *Injection of recombinant juvenile esterase*

Females were anaesthetized on ice and injected into the thorax with 0.5 µl solutions using a glass needle. The dose of JHE is presented in JHE activity units, where 1 unit is 40 pmol of JH-III hydrolysed per minute. Control injections were done with Grace's medium containing the same concentration of sodium azide that was present in the JHE injections or with BSA using equivalent protein concentrations than rJHE. Less than 5% mortality was observed 24 h after the injection of rJHE or BSA. In contrast the injection of low doses of rJHE-mutant resulted in very high levels of mortality by 24 h; nevertheless, survival was normal 1 h after the injection, therefore we have been able to use the rJHE-mutant in short-term experiments. The insecticide activity of the JHE-SG mutant on *H. virescens* has been previously described (Bonning *et al.*, 1995).

### *Haemolymph collection*

Haemolymph was collected by removing the legs at the coxo-femoral joints, and the bodies were placed in a double tube (a small hole was made at the bottom of the inner tube holding the mosquito) and centrifuged at 2500 g for 2 min.

### *Juvenile hormone esterase assay*

Esterase activity was measured using a partition assay described by Hammock & Sparks (1977) using <sup>3</sup>H-JH-III as a substrate with the modification that toluene was used for organic extraction.

### *RNA isolation and characterization*

Total RNA was isolated from whole mosquitoes using RNA binding glass powder as previously described (Noriega & Wells, 1993). RNA was separated by electrophoresis on 1.2% agarose gels under denaturing conditions using the formaldehyde method

(Fourney *et al.*, 1988). RNA was transferred to Nytran (Schleicher & Schuell, Keene, NH) and hybridized with an ET cDNA (Noriega *et al.*, 1996b), labelled using the Random Prime Labelling System (BRL, Gaithersburg, MD) and  $\alpha$ -<sup>32</sup>P-dATP (ICN, Irvine, CA). Hybridizations were performed under high-stringency conditions, as previously described (Noriega & Wells, 1993). The amount of radioactivity bound to individual samples on the filter was quantified using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Background in all filters was less than 1%.

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