Recombinant Juvenile Hormone Esterase as a Biochemical Anti-Juvenile Hormone Agent: Effects on Ovarian Development in *Acheta domesticus*

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By investigating the effects of recombinant juvenile hormone esterase (JHE) on the stimulation of ovarian development and egg laying in the house cricket *Acheta domesticus* L., we have tested the hypothesis that recombinant JHE (derived from the tobacco budworm *Heliothis virescens*) can be used as a biochemical anti-juvenile hormone (JH) agent. Recombinant JHE, produced by a genetically engineered baculovirus, was affinity-purified and injected into females of *A. domesticus.* JHE was cleared rapidly from the hemolymph of the crickets. However, upon repeated injection, significant reductions were seen in the extent of development of the ovaries and in the numbers of eggs laid. The effects of JHE could be rescued by topical application of the JHE inhibitor, OTFP. Thus, we have demonstrated an anti-JH effect on reproduction and that the recombinant JHE derived from a lepidopteran is active in an orthopteran insect. Arch. Insect Biochem. Physiol. 34:359–368, 1997.   © 1997 Wiley-Liss, Inc.

Key words: recombinant JHE; *Acheta domesticus;* JHE pharmacokinetics; vitellogenesis

INTRODUCTION

Testing for the involvement of juvenile hormone (JH[†]) in a physiological process is a common goal of insect physiologists. Removal of the corpora

allata which synthesize the JH often is technically difficult and can result in severe trauma to the animal. Chemical anti-juvenile hormones have proven very valuable but are not universally active among insect groups and often have other pharmacological and toxicological effects. Since the juvenile hormone esterase (JHE) from *H. virescens* hydrolyzes the known JHs of most insects very rapidly, we tested the hypothesis that it could function as a biochemical allatectomizing agent. For this we used the well-studied stimulation of ovarian development and egg laying in the house cricket, *Acheta domesticus.*

Juvenile hormone plays a critical role in coordination of events leading to vitellogenesis in many insects (Engelman, 1979; Hagedorn and Kunkel, 1979). In some insects, JH is necessary for production of the precursor protein vitellogenin by the fat body, for accumulation of this protein within the oocytes, and for subsequent oocyte maturation. In other insects such as mosquitoes, JH is necessary for previtellogenic events only: exposure of the fat body and ovary to JH is required before vitellogenin can be synthesized or stored (Flanagan and Hagedorn, 1977; Hagedorn et al., 1977), and exposure to JH leads to formation of the endocytic complex needed for oocyte competence to internalize proteins (Raikhel and Lea, 1985). Several other aspects of ovarian previtellogenic development in insects are also controlled by JH, such as growth of follicles and differentiation of the follicular epithelium. In locusts JH stimulates synthesis of vitellogenin as well as uptake into the oocytes (Chen et al., 1976; Ferenz and Kaufner, 1981; Irvine and Brasch, 1981).

The house cricket, *A. domesticus,* has 160 ovarioles which develop asynchronously. Oocyte maturation is complex, with overlap of the maturation cycles for different groups of eggs (Renucci et al., 1985, 1987). From 60–100 eggs are laid per day beginning on day 4 after the imaginal molt. The sharp peak of JH on day 1 is associated with previtellogenesis. There is also a sharp peak of JH on day 3, and the amount of vitellogenin in the plasma surges (Bradley and Edwards, 1978). This elevated JH titer is correlated with the onset of vitellogenesis and oogenesis. The only JH homolog detected so far in a number of different Orthoptera (Loher et al., 1983) and in the plasma of adult *A. domesticus* (Strambi et al., 1984) is JH III.

The titer of JH in insects is regulated both by the rate of biosynthesis in the corpora allata (Tobe and Pratt, 1975) and by the rate of degradation (Hammock, 1985). Hydrolytic degradation is effected by two classes of enzymes: juvenile hormone esterase (JHE) and epoxide hydrolase (Hammock, 1985). In adult *A. domesticus,* the main route of degradation of JH appears to be via JHE (Woodring and Sparks, 1987), which is produced by the fat body (Renucci et al., 1984). The titer of JHE is correlated with the titer of JH at the beginning of oogenesis (Renucci et al., 1984). During the early stages of embryogenesis, the titer of JH is relatively high to promote yolk deposition (Renucci and Strambi, 1983). Subsequently, however, low titers are maintained in the eggs until after blastokinesis (Roe et al., 1987a,b). The elevated titers of JHE in preovipositional and newly laid eggs may be required for removal of maternal JH from the egg (Roe et al., 1987a).

The titer of JHE during the first 3 days of a newly emerged *A. domesticus* is relatively constant at about 6 nmol JH/mg-min, with a sharp peak on day 4 (Renucci et al., 1984). A correlation between JHE activity and JH titer has

been established through the first 18 days of adult life for females, with JHE activity being high when JH titers are low and vice versa (Woodring and Sparks, 1987).

For production of recombinant JHE, the cDNA sequence encoding JHE was derived from the tobacco budworm *Heliothis virescens* (Hanzlik et al., 1989) and inserted into a recombinant baculovirus (Bonning et al., 1992; Hammock et al., 1990). High levels of JHE are produced by the recombinant baculovirus on infection of cultured insect cells and are exported into the culture medium. Here we demonstrate the use of recombinant JHE as a powerful anti-JH agent: injection of JHE into *A. domesticus* results in an inhibition of vitellogenesis.

MATERIALS AND METHODS

Insects

House crickets, *Acheta domesticus,* were purchased from Fluker's Cricket Farm (Baton Rouge, LA) and were reared in the laboratory of UC Berkeley at 27°C on an LD cycle of 12:12 h. Nymphal instars were held in 50 liter garbage cans, whereas nymphs from the last instar and adult crickets were individually isolated in 50 ml glass vials with perforated snapcap lids. The insects were fed daily with fresh romaine lettuce and pellets of Purina mouse chow.

Production and Purification of Recombinant JHE

Recombinant JHE derived from the lepidopteran *Heliothis virescens* (Hanzlik et al., 1989) was produced by infection of the cell line Tn5B1-4 "High Five" (Invitrogen, La Jolla, CA) with the recombinant baculovirus AcUW2-(B)JHE (Bonning et al., 1992). Cells were maintained in the medium Excell 401 (JRH Biosciences, Lenexa, KS) containing 1% penicillin/streptomycin and were shaken at 100 rpm in Erlenmeyer flasks. Cells were infected at a multiplicity of infection of between one and five. Three days postinfection, cells and medium were harvested, and recombinant JHE was purified from the medium by affinity chromatography, using 3-(4-mercaptobutylthio)-1,1,1-trifluoro-2-propanone (MBTFP) linked to Sephadex gel and 3-*n*-pentylthio-1,1,1-trifluoro-2-propanone (PTFP) as eluant, as described previously (Shiotsuki et al., 1994), with the modification that the cell culture medium was diluted 1:2 with buffer (30 mM sodium phosphate buffer, pH 7.4, 1.5% sucrose, 0.01% sodium azide) prior to loading onto the column. The affinity-purified JHE used for injections was suspended in 0.1 M phosphate buffer, pH 7.4, containing 5% sucrose (PBS).

Pharmacokinetics of Recombinant JHE in *Acheta domesticus*

Affinity-purified recombinant JHE was injected in a volume of 5 μl into the abdomen of cold-anesthetized females of *A. domesticus* 1 day after the imaginal molt. A sample of 2.7 units was injected, where one unit of JHE is capable of hydrolyzing 1 μmole of methyl 1-heptylthioacetothioate (HEPTAT) per minute (McCutchen et al., 1993), which is equivalent to hydrolyzing 20 nmol of [³H]JH III per minute (McCutchen et al., 1995). The specific activity for JHE is approximately 4.5 μmol [³H]JH III per minute/milligram. Hemolymph

was collected at 10, 15, 20, 30, 60, and 120 min after injections from three to eight different specimens per time point. Each insect was bled only once. Hemolymph was diluted 1:25 in sodium phosphate buffer (pH 7.4, I = 0.2) containing 5% sucrose, 0.01% phenylthiourea, 0.02% sodium azide, and 0.0025% Triton X-100 and was frozen prior to analysis. Hemolymph samples were also taken from uninjected crickets and treated in the same way. The experiment was replicated at a separate time.

Assay of Hemolymph for JHE Activity

Hemolymph samples were thawed and assayed for JHE activity by using the chromogenic substrate HEPTAT (McCutchen et al., 1993). Catalytic activity resulting from nonspecific esterases present in the hemolymph was accounted for by repeating the assay in the presence of the trifluoromethylketone, 3-n-octylthio-1,1,1-trifluoro-2-propanone (OTFP) (McCutchen et al., 1995). Positive readings for assays in the presence of OTFP were deducted from values obtained for assays in the absence of OTFP. The half-life of injected recombinant JHE in *A. domesticus* was calculated by exponential regression analysis using the Statgraphics® statistical program.

Effects of Recombinant JHE on Ovarian Development

Seven female *A. domesticus* were anesthetized in crushed ice and injected with 1.7 units of recombinant JHE in a volume of 10 μl six times over a period of 10 days beginning on the day of the imaginal molt. The crickets were given the opportunity to mate on two occasions and were provided with fine moist sand for oviposition. Females that mated were dissected when 13 days old, and the number of eggs was counted. Oviposition of four control insects of the same age was monitored.

In a second experiment, three groups of four to six females of the same age (±1 day) were injected six times with 2.5, 5, or 10 units of recombinant JHE in a volume of 10 μl. A fourth group was injected with PBS in the same manner. Injections were carried out beginning on the day of the imaginal molt and then every other day. At the age of 12–14 days the females were mated, provided with sand for oviposition during a period of 24 h, and then dissected. The ovaries were removed, weighed in the wet state, dried, and weighed again. Data were analyzed by fewest squares means analysis.

Reversal of the Effects of JHE on Ovarian Development by Application of a JHE Inhibitor

In order to reverse the effects of injected JHE on ovarian development in *A. domesticus*, OTFP was applied topically following injection. Treatments began on the day of the imaginal molt, with three to seven crickets for each treatment. Controls were injected with 5 μl of PBS every other day. Controls for OTFP treatments were topically treated with 0.01 M OTFP in acetone: 3 μl every other day (21 μl total per female) or every other day six times and then daily for the next 6 days (total of 36 μl per female). Test insects were injected with 1 unit of recombinant JHE in a volume of 5 μl every other day for 12 days (total of 30 μl) and were then dissected. Insects in a second test group were injected with JHE in the same way and then treated topically with 3 μl

of 0.01 M OTFP (total of 18 μl). The total number of eggs produced (i.e., laid or stored in the ovaries) by each group was counted. The age of the females at the end of the various trials was 15–18 days. Data were analyzed by ANOVA with transformation to stabilize variance.

RESULTS AND DISCUSSION

Pharmacokinetics

The half-life of recombinant JHE in the hemolymph of *A. domesticus* was 30.5 min (Fig. 1). JHE activity in uninjected crickets was 0.4 ± 0.01 units per milliliter of hemolymph. The maximum amount of JHE in the hemolymph immediately following injection was 9 units/ml hemolymph. This is equivalent to approximately ten times the maximum titer of JHE normally seen in a cricket (Woodring and Sparks, 1987). When hemolymph samples were assayed in the presence of OTFP, JHE activity levels were less than 20% of those for samples assayed in the absence of OTFP. This indicates that non-specific esterases contributed relatively little to the esterase activity detected by using the substrate HEPTAT.
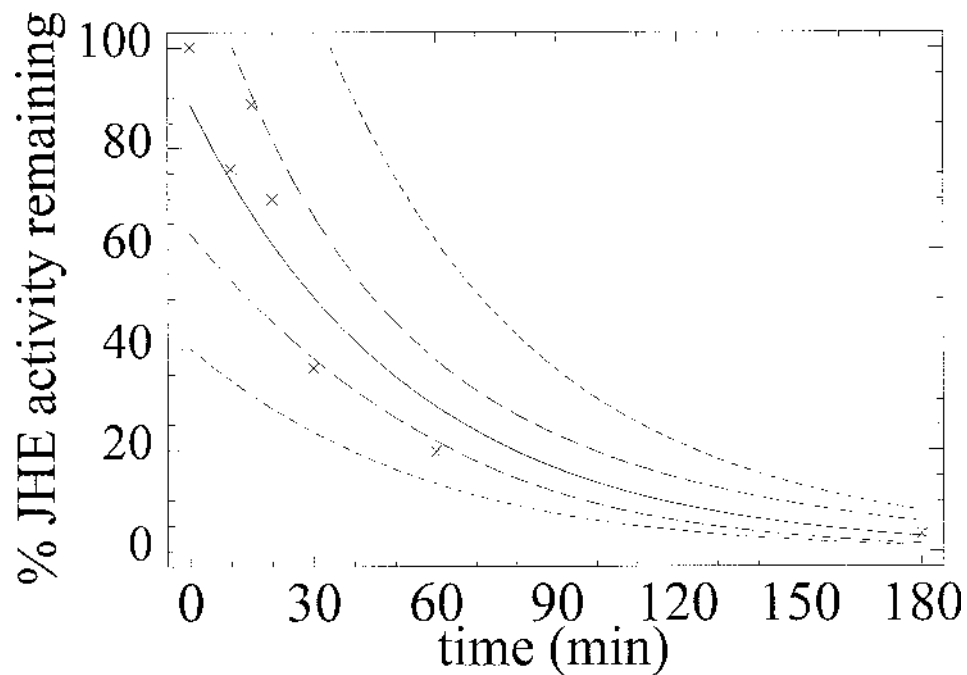

Fig. 1.   Clearance of recombinant JHE from the hemolymph of female *A. domesticus.* Recombinant JHE was injected into the abdomens and hemolymph was collected from three to eight crickets at each time point after injection. Each insect was only bled once. Broken lines represent prediction (inner lines) and confidence limits (outer lines) at the 95% confidence level. Prediction limits are based on estimates of values of the dependent variable for selected values of X, based on the model Y = exp(4.485 − 0.0188X). The correlation coefficient for the graph is 0.98.

In Lepidoptera, JHE is removed from the hemolymph by a discrete organ, the pericardial cell complex, where it is presumed to be degraded (Booth et al., 1992; Ichinose et al., 1992a,b). Whether this is also true for crickets is difficult to ascertain, because their pericardial cells are dispersed throughout the fat body tissue. In any case, removal of the recombinant JHE from the cricket hemolymph was very rapid, indicating that repeated injection of JHE was necessary to see any anti-JH effect on ovarian development. For all experiments, the total amount of recombinant JHE was in excess of the normal maximum JHE titer seen during the last instar (Woodring and Sparks, 1987).

Effects of Recombinant JHE on Ovarian Development

Following injection of female crickets with recombinant JHE after the imaginal molt, few proceeded to mate. Only two of the seven crickets injected with recombinant JHE mated. These two were dissected and had an average of 78 ± 50 eggs. These eggs were black and brown in color, partially empty, and in a state of resorption at 13 days. The second and third order of oocytes had
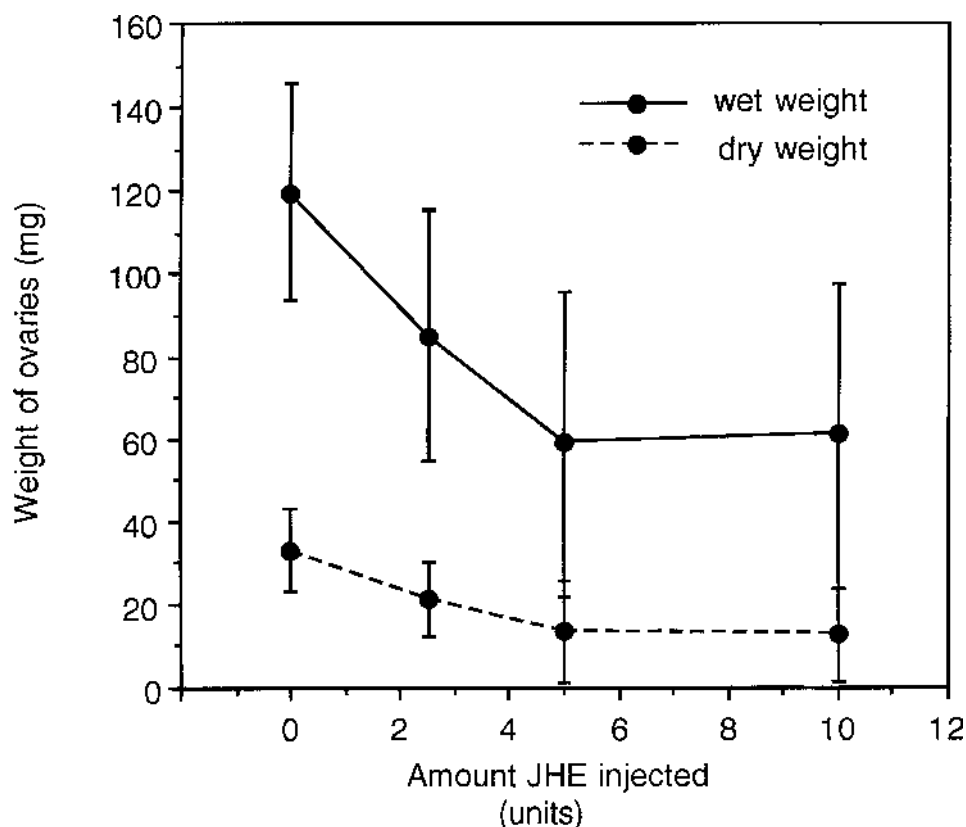

Fig. 2. Effect of recombinant JHE on ovarian development. Wet and dry weights of ovaries were recorded following injection of female crickets after the imaginal molt. The weights of the ovaries from crickets injected with five or ten units of JHE were significantly lower than the weights of ovaries from untreated control insects ($P < 0.05$).

developed very little. The four control insects of the same age produced an average of 460 ± 30 eggs. Thus, insects injected with JHE produced 83% fewer eggs on average compared to the control crickets. This supports the premise of the importance of JH for ovarian development.

For the second experiment, the weights of the dissected ovaries were lowest for those insects injected with the highest concentration of JHE (Fig. 2), and the development of the ovaries was inversely related to the amount of recombinant JHE injected. Least squares means analysis showed that both wet and dry weights of ovaries from crickets injected with five or ten units of JHE were significantly less than ovaries for the uninjected controls ($P < 0.05$). The numbers of eggs laid were low, as the females were given only one opportunity to oviposit at the end of the experiment. The developed eggs were brown in color and had stuck together, which allowed eggs and ovaries
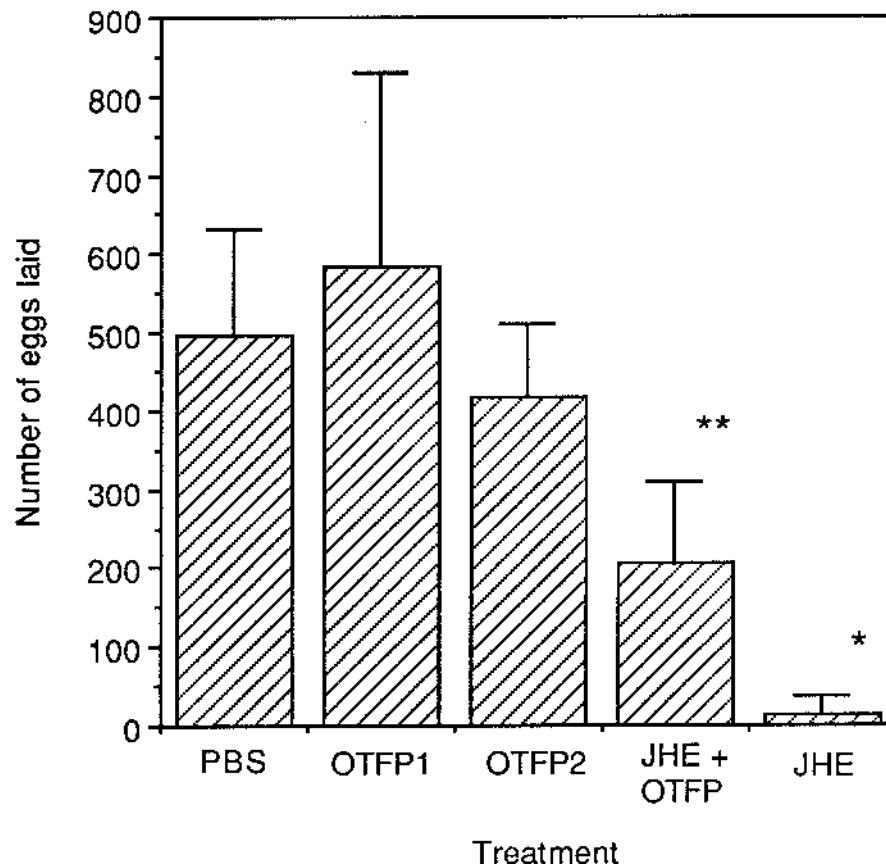

Fig. 3.   Reversal of the anti-JH effects of recombinant JHE with the JHE inhibitor OTFP. Crickets were injected with 1.5 units of recombinant JHE, with or without topical application of a total of 18 μl of 0.01 M OTFP. Control crickets were injected with PBS or dosed with 36 μl or 21 μl of 0.01 M OTFP (OTFP1 and OTFP2, respectively). Injection of JHE resulted in a significant reduction in the numbers of eggs laid compared to (*) injection of PBS or to (**) injection of JHE with topical application of OTFP.

to be weighed together. Examination following dissection showed that high doses of JHE resulted in smaller ovaries with less developed oocytes compared to the ovaries from control insects.

Reversal of the Anti-JH Effects

Injection of recombinant JHE significantly reduced the number of eggs laid compared to the PBS-injected control crickets ($p < 0.05$). The number of eggs laid was reduced by 97% (Fig. 3). This effect was partially reversed (to 41%) by application of the JHE inhibitor, OTFP. The numbers of eggs laid by crickets injected with JHE was significantly lower than those injected with JHE and treated with OTFP (ANOVA; $P < 0.05$). Topical application of either concentration of OTFP on normal crickets had no significant effect on the numbers of eggs laid compared to the PBS-injected controls ($P > 0.05$).

The anti-JH effect of the recombinant JHE on the numbers of eggs laid was only partially reversed by topical application of OTFP. Given that relatively low concentrations of OTFP were used in these experiments, complete reversal of the anti-JH effects may be possible by application of more OTFP. However, reactivation of OTFP-inhibited JHE does occur within the insect (Ichinose et al., 1992a; Philpott and Hammock, 1990).

We have shown that recombinant JHE is a powerful and specific anti-JH reagent when injected into *A. domesticus* beginning on the day of the imaginal molt. Injection of recombinant JHE with or without coapplication of chemical anti-juvenile hormones could be employed as a biochemical mechanism to reduce JH titers. This technology may prove to be a useful supplement to allatectomy in testing the role of JH in physiological processes.

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