

COVER SHEET

TITLE: Analyzing interactions between the GV1 Cell Line, Juvenile Hormone, and Juvenile Hormone Binding Protein

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ABSTRACT

Analyzing interactions between the GV1 Cell Line, Juvenile Hormone, and Juvenile Hormone Binding Protein

Insect juvenile hormone (JH) and its hemolymph transport protein, juvenile hormone binding protein (JHBP) play key roles in the development of many insects. However, the exact mechanism via which JH and JHBP regulates development remains unclear. This study proposes an examination of the molecular interactions between JH and JHBP in *Manduca sexta* GV1 cells and attempts to determine the rate at which GV1 cells metabolize JH to juvenile hormone acid and clarify if JHBP prevents the degradation of JH in vitro.

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I have supervised this work, read this thesis and certify that it has my approval



April 26, 2011

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Abstract

Insect juvenile hormone (JH) and its hemolymph transport protein, juvenile hormone binding protein (JHBP) play key roles in the development of many insects. However, the exact mechanism via which JH and JHBP regulates development remains unclear. This study proposes an examination of the molecular interactions between JH and JHBP in *Manduca sexta* GV1 cells and attempts to determine the rate at which GV1 cells metabolize JH to juvenile hormone acid and clarify if JHBP prevents the degradation of JH in vitro.

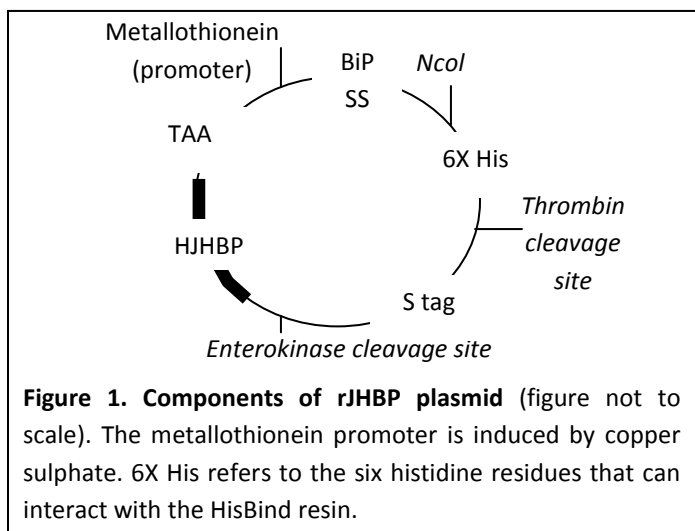
Introduction

Juvenile hormone and binding protein

Juvenile hormones are a family of acyclic sesquiterpenoids that serve as regulators in the normal developmental and reproductive cycles of insects. In particular, JHs regulate the growth of larva and prevent premature metamorphosis. These lipophilic hormones are transported in the insect's circulatory system by hJHBP that protects JH from nonspecific adsorption and enzymatic attack. hJHBP also ensures that an adequate source of JH is available at the target sites when needed (Goodman and Granger 2005). hJHBPs are monomeric low molecular weight proteins (25-35 kDa) and they have been characterized in the insect orders Lepidoptera and Diptera.

Background of study

As native hJHBP is present in low concentrations of 14µg/ml in *Manduca sexta* hemolymph and is difficult to obtain in sufficient quantity and purity, a stable transformed cell line of *Drosophila* S2 cells which produce recombinant JHBP (rJHBP) was established in 2007. This was achieved by cloning the rJHBP

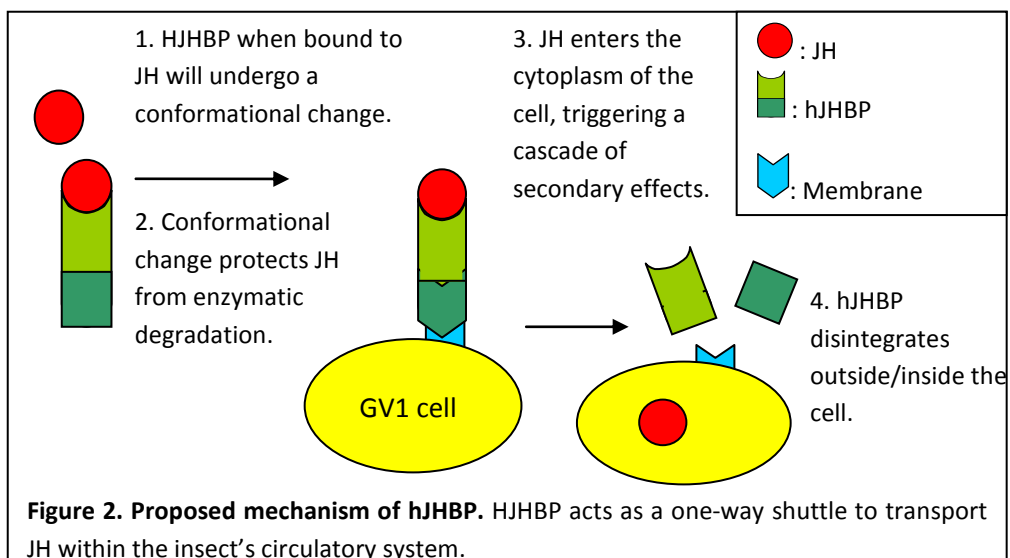


gene into a commercially available vector (Figure 1) and transfecting it into S2 cells. An insect cell line was chosen instead of a bacterial cell line as hJHBP is a glycosylated protein, with carbohydrates accounting for nearly 2 kDa of its molecular weight. Hence, an insect cell line was chosen as bacterial cell lines are not able to produce functionally glycosylated proteins.

The rJHBP is then purified via affinity chromatography containing HisBind resin (Novagen) and used in experiments with GV1 cells from the Lepidopteran, *Manduca sexta* to determine if rJHBP protects JH from enzymatic degradation.

Proposed mechanism

Unbound JH is rapidly inactivated by enzymes naturally present in insect hemolymph and is extremely unlikely to reach target cells in its active form. Hence, it is hypothesized that hJHBP acts as a non-reusable shuttle (Figure 2) that moves JH from the insect's circulatory system into target cells. When JH binds to apo-hJHBP, a conformational change is induced and holo-hJHBP is formed. It is thought that this conformational change shields JH from enzymatic degradation and enables JH to be delivered to target cells in a highly specific and regulated manner.



Hence, the goal of this study is to investigate the first two stages in this process in the GV1 cell line and determine the rate at which JH is degraded and if the addition of JHBP reduces the rate of degradation of JH.

Methods

The GV1 cell line from *Manduca* was recovered from storage in liquid nitrogen and grown in sufficient quantities for the experiments in Grace + 10% fetal bovine serum (FBS) medium. Next [^3H]- JHII was purified via HPLC and stored in toluene at -20°C . The radioactivity, as measured by scintillation counting, was found to be 11000 DPM/ μl . Radioinert JHII was purified by HPLC and brought to 1 mg/ μl in toluene and stored at -20°C .

A control experiment was performed where JH was incubated at room temperature with Grace + 10% FBS medium and Grace + 10% FBS medium which had been pre-incubated with GV1 cells for several

days. It was shown that the Grace + 10% FBS medium and the pre-incubated medium did not contain substances that accelerated the degradation of JH.

To test the rate of degradation of JH by GV1 cells, 6 glass cell culture dishes were containing 5ml of Grace + 10% FBS were seeded with approximately 1,000,000 GV1 cells each and the cells were allowed to grow for 4 days. Cell counts were performed using an automated cell counter (Countess, Invitrogen). Meanwhile, 7.5 µg of rJHBP (500 µl) was obtained and dialyzed in 50 ml of Grace + 10% FBS medium at 4°C overnight to remove the storage buffer which contains imidazole. Approximately 350 µl of rJHBP was recovered after dialysis. Two separate glass vials containing 825,000 DPM radiolabeled JHII and 1.5 mg of radioinert JHII in toluene each were prepared. The toluene was then dried down under N₂ and 330 µl of Grace + 10% FBS was added to one vial while 350 µl of dialyzed rJHBP was added to the other vial. The vials were then incubated at 4°C overnight.

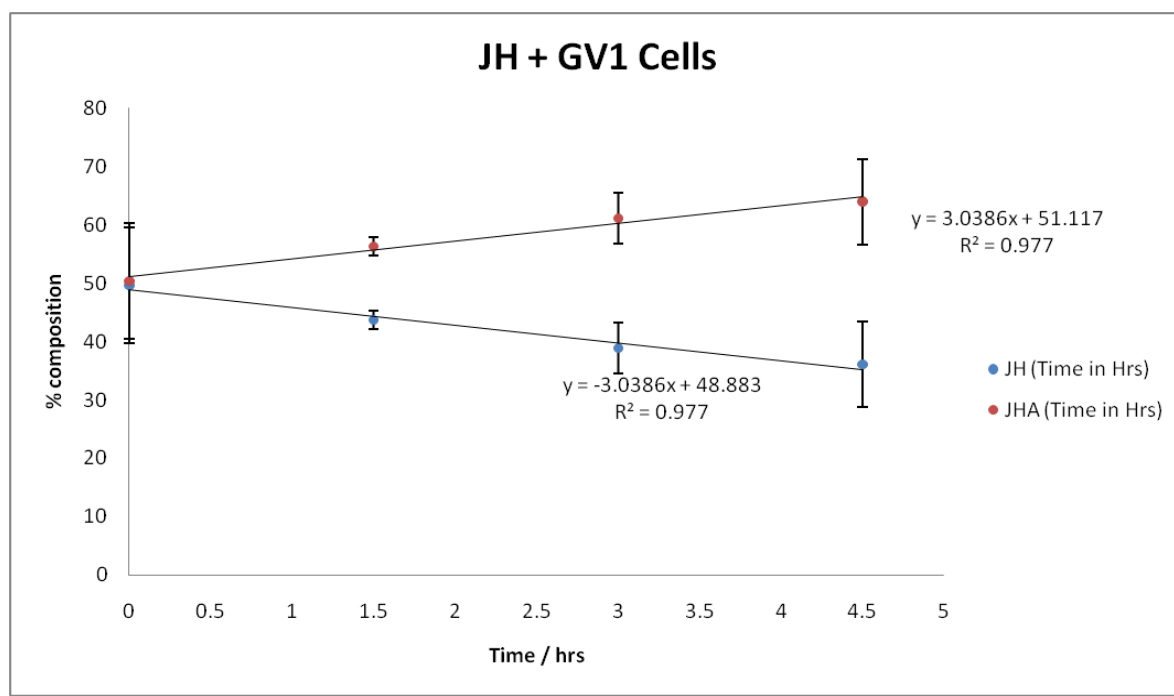
After overnight incubation, 110 µl of JHII, which was pre-incubated with Grace + 10% FBS, was added to three dishes of GV1 cells while 110 µl of JHII + rJHBP, which was pre-incubated with Grace + 10% FBS, was added to another three glass dishes. As a control, 20 µl of JHII + rJHBP, which was pre-incubated with Grace + 10% FBS was added to a glass vial containing 909 µl of Grace + 10% FBS medium. The final concentration of JHII in each culture was calculated to be 100 µg/ml. 200 µl of cell media was removed from each of the samples at 0, 1.5, 3, and 4.5 hours. Each medium sample was then immediately extracted three times with 200 µl of ethyl acetate and dried under N₂. The samples were then stored overnight at 4°C.

The samples were then analyzed using thin layer chromatography (TLC). 25 µl of ethyl acetate was added to each sample vial and the samples were placed onto thin layer chromatography plates with Fast Blue B Stain (SIGMA) as a marker to aide separation of JHII from JHII acid, the primary metabolite of JH. The thin layer chromatography plates were then developed in 95% toluene and 5% ethyl acetate and

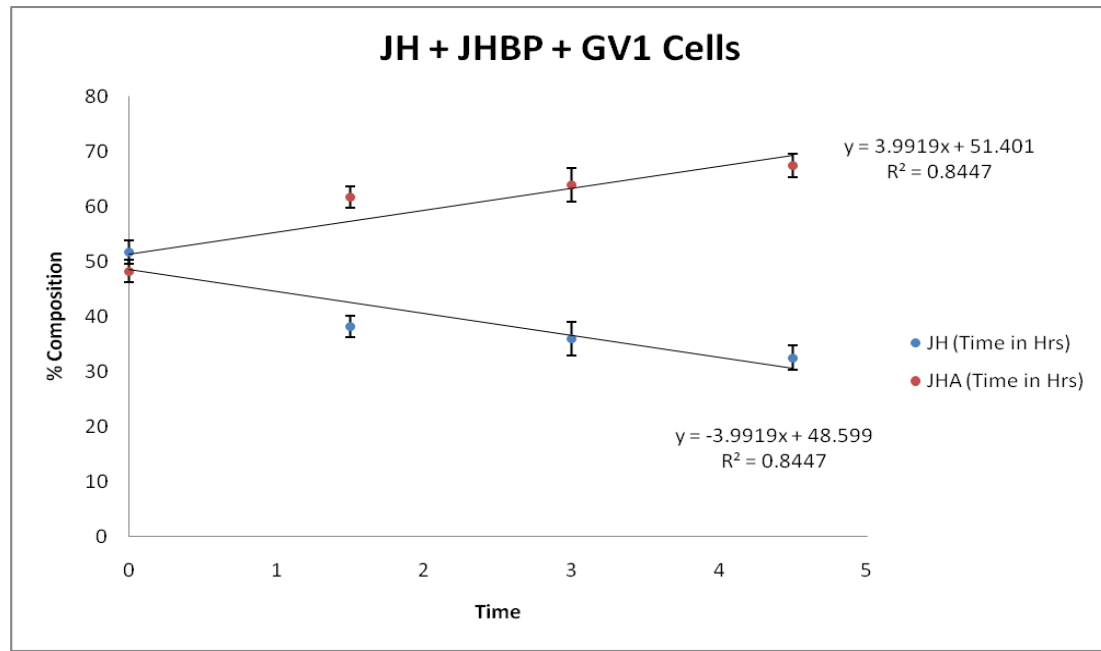
run for approximately 45 minutes until the solvent front was about 3 cm from the end of the plate. The plates were then allowed to dry overnight.

The radioactivity of the samples was then determined using a scintillation counter. Each TLC sample was divided into two portions, the JH and the JHA containing portion. A razor blade was used to scrape each portion into scintillation vials. An equal amount of scintillation fluid was then added to each vial and the vials were vortexed gently before being placed into the scintillation counter.

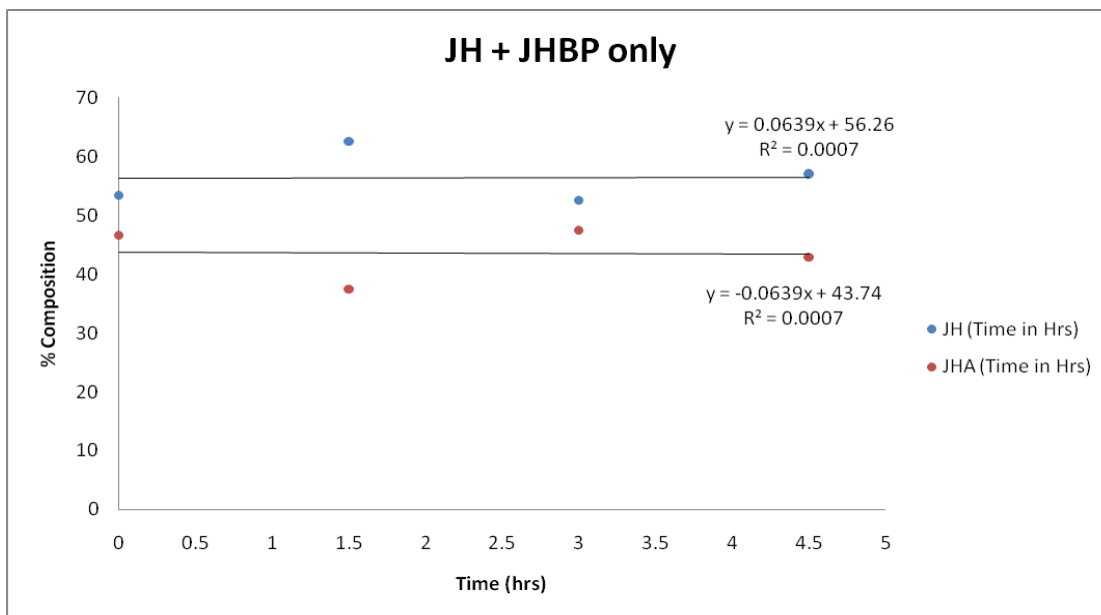
Results



Graph 1. Incubation of JH with GV1 cells in Grace + 10% FBS medium. Graph of percentage composition of JH and JHA vs. incubation time.



Graph 2. Incubation of JH + rJHBP with GV1 cells in Grace + 10% FBS medium. Graph of percentage composition of JH and JHA vs. incubation time.



Graph 3. Incubation of JH + rJHBP with in Grace + 10% FBS medium only. Graph of percentage composition of JH and JHA vs. incubation time.

Discussion and Conclusions

The aim of this thesis is to determine the rate of degradation of JH by GV1 cells and to determine if the presence of rJHBP affects the rate at which GV1 cells degrade JH.

From the experimental data, the rate of degradation of JHII when incubated with GV1 cells was determined to be 3.04%/hr (3.04 µg/hr). The rate of degradation of JHII when incubated with GV1 cells with rJHBP present was determined to be 3.99%/hr (3.99 µg/hr). The rate of degradation of JHII with Grace + FBS medium only was determined to be 0.06%/hr (0.06 µg/hr). By performing a two way analysis of variance and a pairwise multiple comparison (Bonferroni t-test), it was determined that there was insufficient data to prove that the rate of degradation of JHII by GV1 cells was significantly different when rJHBP was present. However, the rate of degradation of JHII by GV1 cells was significantly different when compared to the control which did not contain GV1 cells.

Possible future improvements to the experiment include. A higher concentration of rJHBP should be used relative to the JHII present to ensure that most of the JHII is bound to rJHBP. A longer incubation period could be performed as more significant differences may have been detected over a longer time period. Finally, more replicates could be conducted to reduce the sample standard deviation.

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