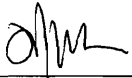# ABSTRACT

Stable expression and purification of Juvenile Hormone Binding Protein from *Drosophila melanogaster* Schneider line-2 cells

Studies on the hemolymph Juvenile Hormone Binding Protein (hJHBP) and Juvenile Hormone (JH) interaction have been considerably limited by the availability of pure hJHBP. An expression vector was created by cloning the recombinant *JHBP (rJHBP)* gene into pMT/BiP/V5-HisB vector and stably transfecting it into *Drosophila melanogaster* Schneider line-2 cells. The cells can be induced by copper sulfate to produce large quantities of rJHBP that is secreted into the medium. The rJHBP can be purified by passing it through a His·Tag column. Subsequent treatment with recombinant Enterokinase yields a protein that is similar to hJHBP in size. Results from radioactive binding assay affirm the functionality of rJHBP because it is able to bind JH rather efficiently. These suggest that rJHBP is folded and glycosylated in a manner similar to that of native hJHBP in *Manduca sexta*. This is the first successful instance of inducible expression of rJHBP *in vitro*.

Si Hui Tan/Biochemistry
Author Name/Major

Author Signature

17May 2007
Date

Dr. Walter Goodman/Entomology
Mentor Name/Department

Mentor Signature

COVER SHEET

TITLE: Stable expression and purification of Juvenile Hormone Binding Protein from *Drosophila melanogaster* Schneider line-2 cells

AUTHOR'S NAME: Si Hui Tan

MAJOR: Biochemistry

DEPARTMENT: Biochemistry

MENTOR: Dr. Walter Goodman

DEPARTMENT: Entomology

MENTOR(2): Dr. Dave Nelson

DEPARTMENT(2): Biochemistry

YEAR: 2007

(The following statement must be included if you want your paper included in the library's electronic repository.)

Abstract

Studies on the hemolymph Juvenile Hormone Binding Protein (hJHBP) and Juvenile Hormone (JH) interaction have been considerably limited by the availability of pure hJHBP. An expression vector was created by cloning the recombinant *JHBP (rJHBP)* gene into pMT/BiP/V5-HisB vector and stably transfecting it into *Drosophila melanogaster* Schneider line-2 cells. The cells can be induced by copper sulfate to produce large quantities of rJHBP that is secreted into the medium. The rJHBP can be purified by passing it through a His·Tag column. Subsequent treatment with recombinant Enterokinase yields a protein that is similar to hJHBP in size. Results from radioactive binding assay affirm the functionality of rJHBP because it is able to bind JH rather efficiently. These suggest that rJHBP is folded and glycosylated in a manner similar to that of native hJHBP in *Manduca sexta*. This is the first successful instance of inducible expression of rJHBP *in vitro.*

Introduction

The juvenile hormones (JH) are central in insect development and reproduction, including metamorphosis, insect migratory behavior and color changes (Goodman W.G. 2005). To exert its regulatory effects, the hormone must be distributed throughout the insect circulatory system. However, the lipophilic nature of JH presents a problem. Target site distribution of the hydrophobic hormone is difficult due to nonspecific binding in the circulatory system. Furthermore, the O-methyl ester at the C1 position of JH is highly susceptible to degradation by hemolymph (blood) esterases to yield a nonfunctional molecule.

To overcome these problems, JH is transported bound to a specific hemolymph juvenile hormone binding protein (hJHBP). In addition to preventing JH adsorption and enzymatic hydrolysis, hJHBP also maintains a steady reservoir of JH in the hemolymph that tissues can tap on readily (Goodman 1985; Goodman, Coy et al. 1990; Goodman W.G. 2005).

The hJHBP/JH interaction is specific and high affinity ($K_D=10^{-9}$M). In *Manduca sexta*, the experimental animal, more than 99% of JH is found in the hJHBP-JH complex (holo-hJHBP). However, less than 1% of the hJHBP is loaded with JH (Hidayat and Goodman 1994). Hence, an equilibrium that strongly favors the formation of hJHBP-JH complex is present, while free JH is virtually absent, since any free JH is rapidly degraded by esterases (Roe and Venkatesh 1990).

The exact mechanism(s) by which JH elicits downstream effects in the signal transduction pathways is not known. There are many possible modes of action of JH which may be neither strictly that of peptide or steroid, in the face of the blurring distinction between these two well-known modes of hormone action (Loof 1987). Nevertheless, the lack of free JH and the high affinity of hJHBP for JH together suggest that hJHBP may play a pivotal role in

facilitating the recognition or uptake of JH by target cells through binding JH. Studies of JH and hJHBP in *Galleria mellonella* revealed that the binding of JH to its transport protein alters its sedimentation coefficient, and they concluded that the association induced a conformational change of hJHBP, which could play a role in signal transmission (Wieczorek and Kochman 1991). Preliminary studies with JH and hJHBP in *Manduca sexta* agree with Wieczorek and Kochman's hypothesis, though it remains to be confirmed.

One possible mechanism of JH recognition by the target cell is similar to that of a peptide hormone. Membrane receptors on the cell surface have to detect, recognize and bind JH loaded onto hJHBP. As there is abundant unloaded hJHBP (apo-hJHBP) in the hemolymph, membrane receptors of target cells should be able to discern between the holo- and apo- forms of hJHBP so that only the loaded hJHBP will trigger a response from the cell. The membrane receptors of the target cells in *Manduca sexta* probably recognize holo-hJHBP by the conformational change induced in hJHBP by the binding of JH.

To conduct further studies on the interaction between JH and hJHBP, milligram quantities of *Manduca sexta* hJHBP are required. While the procedure for purification of JH and its analogs by High Performance Liquid Chromatography has been outlined (Cusson, Miller et al. 1997), expression of JHBP in its native conformation in *Manduca sexta* has not been acomplished. It is postulated that recombinant JHBP (rJHBP) expressed in Schneider line-2 cells of *Drosophila melanogaster* (S2 cells) will produce glycosylated hJHBP similar to that in *Manduca sexta.* To prime the S2 cells to produce rJHBP, a construct containing the gene of interest has to be engineered into the pMT/BiP/V5-His vector molecule (Invitrogen). The pMT/BiP/V5-His vector was chosen because it contains the *Drosophila* metallothionein (MT) promoter required for high-level, metal-inducible expression of the gene of interest in S2 cells

(Maroni, Otto et al. 1986; Bunch, Grinblat et al. 1988; Angelichio, Beck et al. 1991). In addition, the vector also contains the *Drosophila* BiP sequence that targets rJHBP protein for secretion, which makes harvesting rJHBP more convenient (Kirkpatrick, Ganguly et al. 1995). The production of the desired protein can be easily induced by adding copper sulfate solution to the medium. The construct will yield a recombinant protein of the following sequence – BiP-6X His-thrombin-S tag-enterokinase-hJHBP (Figure 1).

The *hJHBP*- pMT/BiP/V5-His expression vector will be co-transfected with a plasmid containing the gene encoding for dihydrofolate reductase (DHFR) to allow for selection of the transfected cells with methotrexate (Mtx) to develop a stable cell line (Williams, Poe et al. 1973; Shotkoski and Fallon 1993). Therefore, the objectives of this experiment are to produce a stable S2 cell line expressing rJHBP in a conformation similar to its native one in *Manduca sexta* and purify it for use in conjunction with JH for further studies.
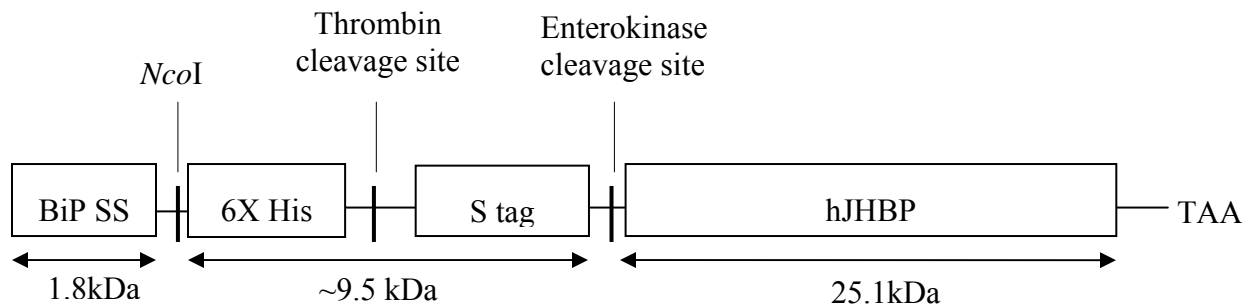
Figure 1. Sequence of rJHBP protein. (*figure not drawn to scale*)

Methods

Construction of Expression vector rJHBP-*pMT/BiP/V5-HisB*

A construct consisting of *rJHBP* in tandem with *Enterokinase* gene was used as the template for *rJHBP* amplification through polymerase chain reaction (PCR). *Nco*I and *Eco*RI

restriction sites were engineered in the forward and reverse primers respectively (forward 5'-gcatccatggtctggttctgg ccatatg-3'; reverse 5'-gcgtgaattccggtaagtcaatagtttgaaaa-3'). The following thermocycling conditions were applied to amplify *rJHBP* from the template using Vent DNA polymerase – two minutes at 94°C; followed by five cycles of 30 seconds at 94°C, 45 seconds at 50°C, and two minutes at 94°C; 30 cycles of 30 seconds at 94°C, 45 seconds at 60°C, and two minutes at 94°C; and lastly, seven minutes at 72°C. PCR results were verified by gel electrophoresis on 1% agarose gel (Figure 2). The amplified *rJHBP* was extracted from the mixture using the Qiaex II Gel Extraction Kit.

Both *rJHBP* and HisB plasmid were digested with *EcoR*I at 37°C for 2.5 hours, then with *Nco*I at 37°C for another two hours. The enzyme-digested products were ligated by incubating them at a 10:1 insert:plasmid ratio, together with T4 DNA ligase at 4°C overnight. A negative ligation control omitting *rJHBP* was also set up to observe the rate of the vector re-ligating upon itself. Successful ligation reactions were selected by transforming the ligation reactions into DH5α cells and plating them on Luria Bertani + Ampicillin (LB+Amp) medium. Fourteen colonies were picked for overnight small-scale inoculation in LB+Amp medium at 37°C. The colonies were screened using the cracking gel analysis to identify those containing larger DNA fragments (Figure 3). Three colonies were selected for further verification by restriction digestion with *Nco*I and *Xba*I at 37°C for two hours. The sizes of the resultant DNA from enzyme digestion were compared to an empty HisB plasmid's (Figure 4). All three colonies were then sequenced using the same primers as those for PCR.

Upon confirmation of the sequence of the *rJHBP*-HisB constructs, one colony was chosen for further inoculation in larger volumes of LB+Amp medium in preparation for use in subsequent transfection. DH5α colonies containing GFP-HisB, HisB and Dihydrofolate

Reductase (DHFR) constructs were also inoculated in larger volumes of LB+Amp medium for the same purpose. The four plasmids were extracted from the media using QIAfilter Plasmid Maxi Kit (Qiagen).

Stable Transfection of Expression Vector into S2 Cells

Two transfection reactions were set up – rJHBP-HisB + DHFR (JHBP) and HisB (without insert).

The S2 cells of *Drosophila melanogaster* were maintained at 27°C in Sf 900-II serum-free medium (GIBCO) and transfected with the two HisB expression constructs and DHFR using Cellfectin (Invitrogen). Cells were seeded in 25cm^2 tissue culture flasks at a density of 2 x 10^6 cells/mL one day before transfection. After overnight incubation, the previous medium was removed. The ratio of expression vector to selection vector transfected was 19:1, the total amount of DNA being 6.25μg per culture. The DNA was diluted with Sf 900-II medium in a total volume of 187.5μL, and the amount of Cellfectin used per culture was optimized to be 62.5 units, which was 10 times as much as the total amount of DNA per culture. Cellfectin was also diluted in 187.5μL of Sf 900-II medium. Both solutions were mixed and incubated at room temperature for 30 minutes. The DNA-Cellfectin complexes were diluted further with an additional 1.4375mL of Sf 900-II medium, all of which (1.875mL) was then inoculated into each cell culture flask containing S2 cells and were incubated for 24 hours. Thereafter, the DNA-Cellfectin-containing medium was replaced with 5mL of new Sf 900-II medium that contains Mtx at a final concentration of 0.5μM. The growth of cells was observed and maintained. The cells were transferred to fresh Mtx-containing medium in a new flask every three to four days. About 1.5 weeks later, discrete colonies resistant to Mtx were observed, and the concentration of

Mtx in the medium was reduced to 0.2μM. Polyclonal stable cell line expressing rJHBP was

obtained after another 2.5 weeks and the cells were cultured to confluency.

Induction of rJHBP Production

Cells were grown to 60-80% confluency, and were induced by adding copper sulfate to

an optimal final concentration of 1mM.   Six days after the addition of copper sulfate, the

medium was harvested and stored in 50% ethylene glycol. rJHBP secretion was tested by

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot. The

medium collected was centrifuged at 300g for 10 minutes to remove any cell remaining in the

medium.

SDS-PAGE and Western Blot Analysis of rJHBP Production and Secretion by S2 cells

Samples were mixed in sample buffer (CONTENTS), boiled for fifteen minutes and

resolved on a 11% SDS-PAGE, followed by transfer of proteins to a nitrocellulose membrane.

The membrane was incubated in blocking buffer (phosphate buffered saline (PBS) solution

(137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4) with 1.5% casein) overnight.

The following day, the membrane was incubated in monoclonal α-hJHBP antibody (Goodman *et*

al., 1990) in blocking buffer for two hours at room temperature, followed by three washes of

five minutes each with washing buffer (PBS with 0.05% Tween 20). The membrane was next

incubated with a secondary antibody, α-mouse-IgG-HRP (KPL Labs, Gaithersburg MD, USA)

for another two hours at room temperature in blocking buffer at 1:1000 dilution, and rinsed with

washing buffer thrice, each wash lasting five minutes. The signal on the membrane was

developed by incubating it with staining solution (0.045M Tris pH 7.6, 0.03% nickel chloride, 0.03% hydrogen peroxide, 2.8mM 3,3'-diaminobenzidine).

JHBP Radioactive Binding Assay

The functionality of the rJHBP expressed by S2 cells was verified with JHBP binding assay. Two dialysis tubings containing either 1mL of harvested medium or 1mL of hemolymph (diluted 20X) from *Manduca sexta* (fifth instar) were incubated in 75mL binding assay buffer (1mM Tris, 100mM potassium chloride, pH 8.0) containing 1μL of ^{3}H-labelled JH II (10R) for 24 hours at 4°C. Scintillation counts of 200μL of the buffer and the samples in both dialysis tubings were measured in duplicates and compared to evaluate JH binding ability of the recombinant JHBP.

Purification of rJHBP from Harvested Medium

The rJHBP secreted by S2 cells was first purified from the harvested medium medium using a column with His·Bind resin (Novagen), which contained nickel ions, to which the six histidine residues on the rJHBP will bind to. The harvested medium in glycerol was repeatedly passed through the His·Bind resin column for one hour at room temperature, and eluted with buffer containing 1M imidazole in 1mL fractions.

Bradford assay was carried out on the eluted fractions and the fractions with the highest protein concentrations were consolidated for dialysis in citrate/borate buffer (30mM citrate, 20mM borate, 5mM dithiothreitol, 40% ethylene glycerol, pH 6.10) overnight at 4°C. The dialyzed solution was concentrated by Microsep 10K Omega size separation membrane (Pall

Corporation) to the range of 0.3-0.4μg/μL). Another Bradford assay was performed to determine

the protein concentration in the final solution.

The rJHBP was cleaved with recombinant Enterokinase (EMD Biosciences) to release

the portion of the protein upstream of hJHBP at a ratio of 1 unit recombinant Enterokinase: 5μg

protein (Entrokinase cleavage site: AspAspAspAspLys$^{\downarrow}$). The Enterokinase was recovered

using the rEKapture kit (EMD Biosciences), and the efficiency of cleavage was analyzed by

SDS-PAGE and Western Blot analysis. The concentration of protein in the eluate from

rEKapture was determined at A_{280}.

Results

*Construction of Expression vector* rJHBP-*pMT/BiP/V5-HisB*

The *rJHBP* gene was amplified for cloning into the pMT/BiP/V5-HisB vector. The size

of the PCR product obtained was approximately 800kb, which corresponded to its expected size
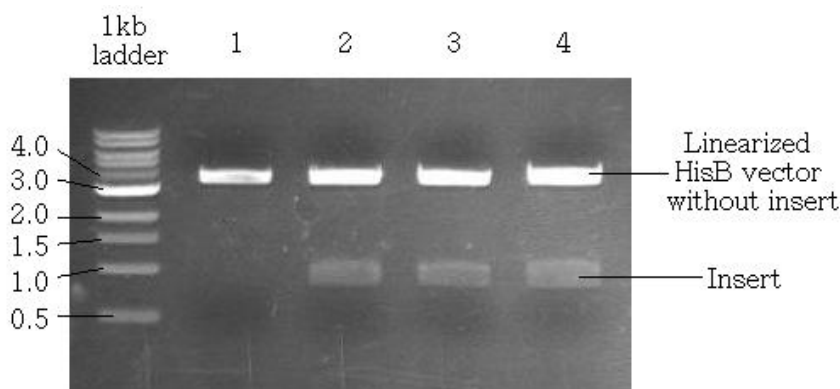
(Figure 2).
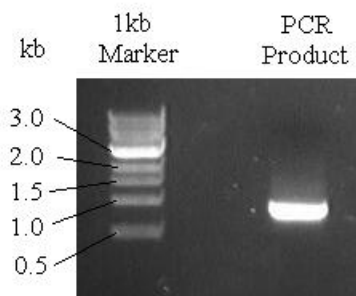
Figure 2. Gel Electrophoresis of PCR product. The expected size of the PCR fragment containing *hJHBP* is approximately 800 kb, which corresponds to the size of the band observed on the 1.0% agarose gel.

The transformation of the ligation reactions indicated that the rate of the vector re-annealing upon itself was low, as shown by the much lower number of colonies observed on the plate with negative control for ligation in comparison to that containing the *hJHBP* insert. Cracking gel analysis suggested that all 14 colonies picked contained the *hJHBP*-pMT/BiP/V5-HisB construct (Figure 3) because the DNA of the *E.coli* colonies consisted of a band

that was significantly larger than the empty pMT/BiP/V5- HisB plasmid (lane 1).   Restriction

enzyme analysis with the three colonies selected showed that they contained the insert (Figure

4). Also, the size of the linearized, digested vector was the same as that of the linearized empty

HisB plasmid (Figure 4). Sequencing of the DNA extracted from the colonies showed that all

three of them contained the expression vector *rJHBP*-pMT/BiP/V5-HisB in the correct reading

frame.
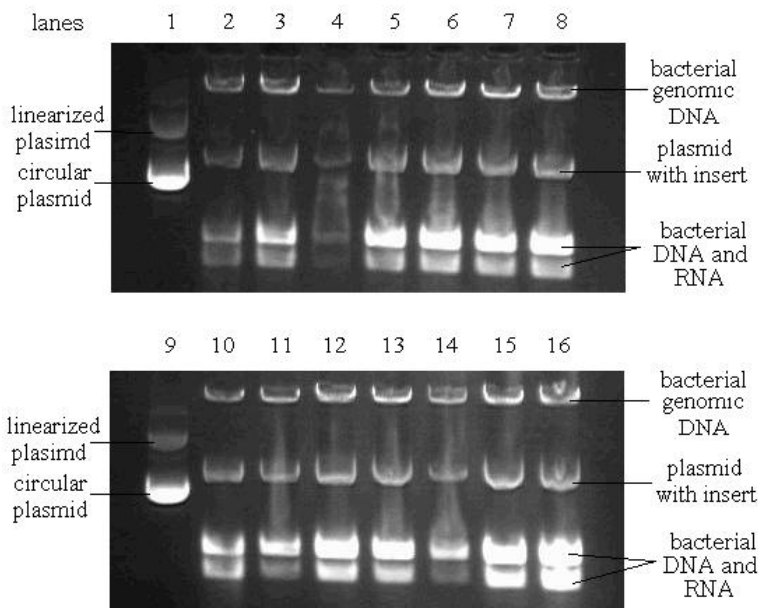
Figure 3. Cracking Gel Analysis.
Fourteen colonies were selected from the
plate of transformed DH5α cells to be
tested for the presence of the vector
ligated with the insert. The bacterial cell
walls were lyzed to release their contents.
The samples were analyzed on a 1%
agarose gel (lanes 2 to 8, 20 to 16)
together with a sample of empty plasmid
(lanes 1 and 9). The results suggest that
all fourteen colonies contain the vector
that has ligated with the insert.

***Figure 4. Verification of vector with restriction
digestion.*** Three of the 14 colonies were selected for
further verification of the presence of the insert in the
vector. The DNA from small scale inoculations of the
three colonies was extracted and digested with *Nco*I
and *Xba*I, along with an empty plasmid. Analysis of
the digestion products on a 1% agarose gel show that
for all three samples (lanes 2 to 4), the linearized
vector with the excised insert is of the same size as the
linearized empty vector (lane 1), and a segment of
DNA that is slightly smaller than 1kb was excised
from the vector. Thus, the vector in the three colonies
contained the insert.

Stable Cell Line Transfection of Expression Vector into S2 Cells

When Mtx was applied, the shape of the S2 cells became elongated, and many of them were observed to either develop processes or appear bloated. The number of surviving cells in both flasks decreased rather drastically after the first few days of transfection. After approximately 1.5 weeks, discrete colonies of non-bloated cells without processes resembling untransfected S2 cells were formed, and they were cultured to confluency four weeks after transfection. The morphology and doubling time of the stable cell lines of rJHBP and HisB were similar to the untransfected S2 cells.

SDS-PAGE and Western Blot Analysis of rJHBP Production and Secretion by S2 cells

Analysis of the media was carried out using SDS-PAGE and Western Blot (Figure 5), along with various other controls as follows. Negative controls comprised of media in which cells treated in one of the following ways were cultured in: untransfected cells, uninduced rJHBP-transfected cells and induced HisB-transfected cells. The positive control was hemolymph from *Manduca sexta* at fifth instar. Media from various flasks that induced rJHBP-transfected cells were grown in were collected for analysis.

The native hJHBP from the hemolymph has a size of 25.1kDa, whereas the expected size for the rJHBP is 36.4kDa, which is close to the apparent size of the rJHBP (~34kDa). Western Blot analysis showed that no rJHBP was present in the media which untransfected cells and induced HiB-transfected cells were grown in, but the uninduced JHBP-transfected cells expressed small amounts of rJHBP. In comparison, the medium with induced rJHBP-transfected cells contained relatively large amounts of rJHBP, indicating the successful expression and secretion of rJHBP by S2 cells.
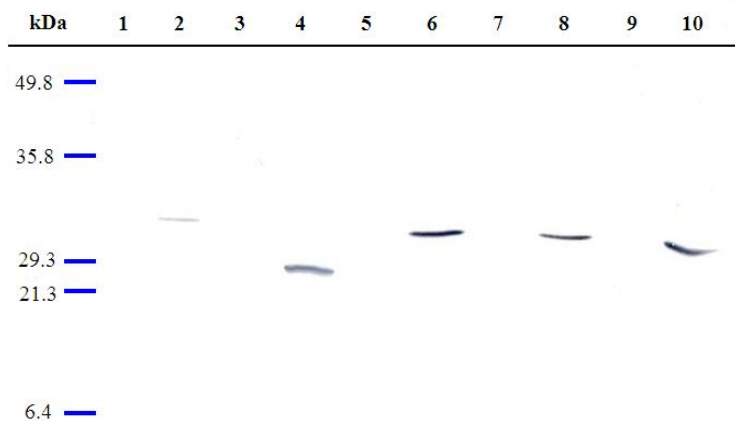
Figure 5. Analysis of expression and secretion of hJHBP by S2 cells. Media in which various transfected and untransfected cells were cultured in were analyzed by SDS-PAGE and Western Blot for presence of hJHBP (detected by a highly specific monoclonal α-hJHBP antibody). The results show that the induced hJHBP-transfected cells expressed and secreted relatively large amounts of hJHBP into the surrounding medium. Controls were media from untransfected S2 cells (lane 1), uninduced hJHBP-transfected cells (lane 2), induced HisB-transfected cells (lane 3), hemolymph (lane 4). Samples of media from three different flasks containing induced hJHBP-transfected cells were also analyzed (lanes 6, 8 and 10). Lanes 5, 7 and 9 were left blank. Ten microliters of each sample was loaded into each well, except for hemolymph (5μL).

JHBP Radioactive Binding Assay

After verification of the expression and secretion of rJHBP by transfected S2 cells, the functionality of the expressed rJHBP was assessed using the JHBP radioactive binding assay. If the JHBP in the dialysis tubing binds JH successfully, it would reduce the concentration of JH in the solution in the dialysis tubing, thus creating a concentration gradient that favors the entry of radioactively-labeled JH from the surrounding binding assay into the dialysis tubing, until equilibrium is reached.

The scintillation results (Table 1) show that the amount of radioactivity in the dialysis tubing containing the medium with rJHBP is more than four times as much that with the 10X diluted hemolymph (contains native hJHBP), indicating that the amount of radioactive JH in the dialysis tubing containing the medium with rJHBP is greater than that containing the diluted hemolymph. The functionality of the rJHBP expressed and secreted by the S2 cells suggests that it can bind JH with reasonably high affinity. This supports the idea that the rJHBP has been folded and processed by S2 post-translational machinery to render it similar to the native hJHBP in the hemolymph.

| Scintillation counts (DPM)	Binding Buffer	Diluted Hemolymph (native hJHBP)	Medium with rJHBP
Sample 1	262.06	402.33	1881.85
Sample 2	281.08	452.54	1860.29
Average	271.57	427.44	1871.07

Table 1: Scintillation results for rJHBP radioactive binding assay. Diluted hemolymph and medium with induced rJHBP-transfected cells were placed in dialysis tubings which were incubated in buffer containing ^{3}H-labeled JH II (10R) for 24 hours and 4°C. Scintillation counts of the buffer, hemolymph and medium with rJHBP were taken in duplicates. The results show that the scintillation results (measured in DPM, disintegrations per minute) for medium with rJHBP are significantly higher than those of binding buffer or the diluted hemolymph, suggesting that the rJHBP secreted by the S2 cells is able to bind JH with relatively high affinity like native hJHBP.

Purification of rJHBP from Harvested Medium

From the Bradford assay, eluted fractions 2 to 4 contained the highest concentration of protein. Subsequently, these fractions were pooled for dialysis in the citrate/borate buffer overnight to remove the imidazole in the elution buffer and store the protein in 40% ethylene glycol. Imidazole was removed from the solution because it inhibits recombinant Enterokinase activity, whereas ethylene glycol was used to store rJHBP because it prevents protein aggregation, thus maintaining integrity of the protein in solution. The dialyzed solution was concentrated using Microsep 10K Omega size separation membrane to a final concentration of 0.36μg/μL.

Various ratios of units of Enterokinase to amounts of protein were experimented and analyzed on SDS-PAGE and Western Blot (1 unit, 0.5 units and 0.2 units of Enterokinase to 10μg of protein, Figure 6, lanes 1 to 3 respectively). A positive control for the efficiency of Enterokinase (Figure 6, lane 4) and a positive control for Western Blot (hemolymph, Figure 6, lane 5) were also included in the experiment. Strikingly, the cleaved rJHBP migrated at a rate

very similar to that of native hJHBP in the hemolymph, indicating that the recombinant protein has been cleaved at the expected site to yield the native hJHBP fragment (Figure 1). The results show that 0.5 units of recombinant Enterokinase is sufficient to cleave 10µg of protein (Figure 6, lane 2). After performing rEKapture, the concentration of protein in the reaction with 0.5 units of Enterokinase:10µg protein (Figure 6, lane 2) was determined to be 0.42µg/µL at A_{280}.
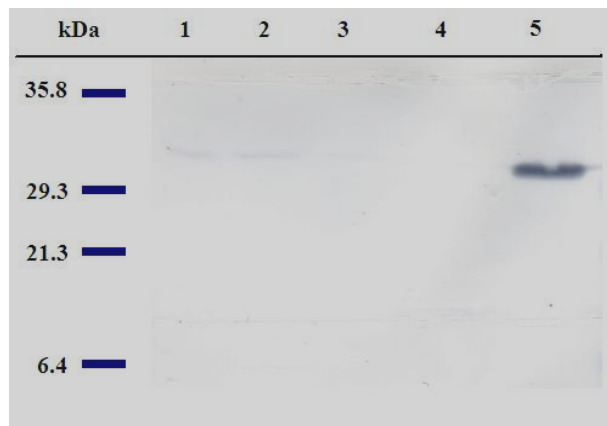
Figure 6. Analysis of efficiency of recombinant Enterokinase cleavage . rJHBP in His·Tag column-purified medium was subjected to Enterokinase cleavage at various Enterokinase:protein ratios – 1 unit:10µg (lane 1), 0.5 units:10µg (lane 2) and 0.2 units:10µg (lane 3). The resulting reactions were analyzed on SDS-PAGE/Western Blot. Hemolymph from *Manuca sexta* in 5th instar was used as the positive control for Western Blot (lane 5). Results showed that 0.5 units of Enterokinase are sufficient for cleavage of the recombinant protein.

Discussion

The aim of the experiment was to create a stable *Drosophila* S2 cell line that expresses rJHBP, and subsequently treat it to yield the protein in its native conformation in *Manduca sexta.* A stable S2 cell line transfected with rJHBP in pMT/BiP/V5-HisB plasmid was shown to express rJHBP, which was secreted into the medium. The recombinant protein could be purified and treated with recombinant Enterokinase to yield hJHBP in its native conformation in *Manduca sexta.*

The vector used in this experiment, pMT/BiP/V5-HisB, did not carry the resistance gene for Mtx. Thus another plasmid containing *DHFR* was co-transfected with the expression vector containing *rJHBP* at a ratio of 1:19. Since S2 cells are unable to differentiate between the plasmids, they will incorporate the plasmids indiscriminately. With a much greater proportion of

HisB plasmid compared to that of *DHFR* in the DNA transfection sample, the probability that the cells that have taken up the *DHFR* plasmid also contains the rJHBP expression vector is greatly increased.   Therefore, selection of cells resistant to Mtx is equivalent to selecting for those that are more likely to express rJHBP.

This is the first instance of inducible and non-toxic expression of JHBP in its native conformation in *Manduca sexta*. Other attempts to express rJHBP in other systems had been relatively unsuccessful. The rJHBP produced in the *E.coli* system was found to bind JH weakly through the JHBP radioactive binding assay, suggesting that the protein was not in its native conformation.   Furthermore, the lack of glycosylation in prokaryotes is likely to be a contributing factor to rJHBP misfolding in *E. Coli* because the mature hJHBP is glycosylated. Expression of rJHBP was also attempted in the *Spodopfeva frugiperda* (Sf211) system with baculovirus (Bonning, Palli et al.). The rJHBP produced in Sf211 was also unglycosylated, and its binding affinity for JH was much lower than that of native hJHBP, indicating that the functionality of the rJHBP produced is lower than that of the latter. Moreover, the expression of rJHBP in the baculovirus system is constitutive and accumulations of large amounts of the protein are deleterious to the host.

Therefore, the S2 system transfected with pMT/BiP/V5-His expression vector established in this study represents the first successful attempt at producing rJHBP that has reasonable binding affinity for JH, suggesting that the rJHBP is in a conformation similar to that of the native hJHBP. Furthermore, as the expression of rJHBP is inducible by copper sulfate, the protein will not accumulate to quantities that are toxic to the cells. Theoretically, when copper sulfate is removed from the medium, the cells will continue to grow as untransfected S2 cells do and stop producing rJHBP; when returned to copper sulfate-containing medium, they will

synthesize large amounts of rJHBP again.

Other advantages of this system are derived from the advantages of the *Drosophila* S2 cells. The cells are semiadherent and do not require addition of trypsin to be lifted into the medium. Also, they do not need to grow in a carbon dioxide incubator nor do they need serum to thrive, reducing possible sources of contamination. Another important advantage of S2 cells is their immortality – there is no limit to how many passages through which the cells can be cultured. Therefore, technically, stable cell lines created can be maintained indefinitely (Muerhoff, Dawson et al. 2004).

Preliminary verification shows that rJHBP produced by S2 cells can be cleaved by Entrokinase to yield a protein that migrates at the same rate as hJHBP in *Manduca sexta* hemolymph, providing support that hJHBP derived from rJHBP has been glycosylated and folded correctly. To obtain sufficient amounts of purified hJHBP for future studies, the recovery of hJHBP from the purification procedure should be further optimized, followed by scaling up rJHBP production and purification.

One of the most pressing questions is the mechanism of how JH binds to hJHBP and the role of hJHBP in JH signaling. The issues can be examined through two approaches. In the first structural approach, the structure of hJHBP can be resolved through crystallography. From the crystal structure, the mechanism of JH binding and the points of contact between hJHBP and JH can be deduced. The hypotheses can be tested with a second, molecular approach. Point mutations and truncations can be made to *hJHBP* gene, inserted into pMT/BiP/V5-His vectors and then expressed in S2 cells. The JH-binding affinities of these mutants and wild-type hJHBP can be compared and analyzed for necessity of certain structural regions or amino acids using the JHBP radioactive binding assay. Also, the postulated conformational change that hJHBP

undergoes when JH binds to it can be inferred by looking at trypsin digestion products of JH-loaded hJHBP and unloaded hJHBP. If JH binding induces a conformation change in hJHBP, there would be some differences in the products of both trypsin digestions. The differences in the sites of the trypsin cleavages can allude to the positions of conformational changes that hJHBP had undergone. Subsequently, these positions can be corresponded to those in the crystal structure to visualize the conformational changes of hJHBP when JH binds to it.

Understanding hJHBP's conformational change when JH is bound to it may shed light on the molecular mechanism of the interaction between JH, its transport protein and the target cell. This information can be applied in insect pest control by synthesizing peptide fragments that block JH's action at target sites. Furthermore, the mechanism of how JH and hJHBP initiate signal transduction in cells would be interesting to find out because it may add to the conundrum of signal transduction pathways that have elements of both peptide and steroid hormone pathways.

References

Angelichio, M. L., J. A. Beck, et al. (1991). "Comparison of several promoters and polyadenylation signals for use in heterologous gene expression in cultured Drosophila cells." Nucleic Acids Res **19**(18): 5037-43.

Bonning, B. C., S. R. Palli, et al. Purification and Reassessment of Ligand Binding by the Recombinant, Putative Juvenile Hormone Receptor of the Tobacco.

Bunch, T. A., Y. Grinblat, et al. (1988). "Characterization and use of the Drosophila metallothionein promoter in cultured Drosophila melanogaster cells." Nucleic Acids Res **16**(3): 1043-61.

Cusson, M., D. Miller, et al. (1997). "Characterization of antibody 444 using chromatographically purified enantiomers of juvenile hormones I, II, and III: implications for radioimmunoassays." Anal Biochem **249**(1): 83-7.

Goodman W.G., G. N. A. (2005). The Juvenile Hormones. Comprehensive molecular insect science Volume 3. L. I. Gilbert, Latrou K., Gill S.S, Elsevier Pergamon**:** 320-340.

Goodman, W. G. (1985). Relative hemolymph juvenile hormone binding capacity during larval, pupal and adult development in Manduca sexta, Pergamon Press. **15:** 557-564.

Goodman, W. G., D. C. Coy, et al. (1990). "Development and application of a radioimmunoassay for the juvenile hormones." Insect Biochemistry **20**(4): 357-364.

Hidayat, P. and W. G. Goodman (1994). Juvenile hormone and hemolymph juvenile hormone binding protein titers and their interaction in the hemolymph of fourth stadium Manduca sexta, Elsevier Science. **24:** 709-715.

Kirkpatrick, R. B., S. Ganguly, et al. (1995). "Heavy chain dimers as well as complete antibodies are efficiently formed and secreted from Drosophila via a BiP-mediated pathway." J Biol Chem **270**(34): 19800-5.

Loof, A. (1987). The impact of the discovery of vertebrate-type steroids and peptide hormone-like substances in insects, Springer. **45:** 105-113.

Maroni, G., E. Otto, et al. (1986). "Molecular and cytogenetic characterization of a metallothionein gene of Drosophila." Genetics **112**(3): 493-504.

Muerhoff, A. S., G. J. Dawson, et al. (2004). Enzyme-Linked Immunosorbent Assays Using Recombinant Envelope Protein Expressed in COS-1 and Drosophila S2 Cells for Detection of West Nile Virus Immunoglobulin M in Serum or Cerebrospinal Fluid, Am Soc Microbiol. **11:** 651-657.

Roe, R. M. and K. Venkatesh (1990). Metabolism of juvenile hormones: degradation and titer regulation, Rutgers University Press. **1:** 126-179.

Shotkoski, F. A. and A. M. Fallon (1993). "The mosquito dihydrofolate reductase gene functions as a dominant selectable marker in transfected cells." <u>Insect Biochem Mol Biol</u> **23**(8): 883-93.

Wieczorek, E. and M. Kochman (1991). "Conformational change of the haemolymph juvenile-hormone-binding protein from Galleria mellonella (L)." <u>Eur J Biochem</u> **201**(2): 347-53.

Williams, M. N., M. Poe, et al. (1973). "Methotrexate binding to dihydrofolate reductase from a methotrexate-resistant strain of Escherichia coli." <u>J Biol Chem</u> **248**(18): 6375-9.