

CLONING, EXPRESSION AND BIOLOGICAL ACTIVITY OF THE JUVENILE HORMONE ESTERASE FROM *Heliothis virescens*

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INTRODUCTION

One of the more exciting trends in science is the increasingly short space of time between many fundamental discoveries and their impact on applied science. In turn, applied problems increasingly can be addressed directly by fundamental approaches. While solving such applied problems one often sees an advancement of the fundamental field as well. These trends certainly are illustrated by our recent work on the juvenile hormone esterases (JHE's) of lepidopterous larvae. Expression of this enzyme in several baculovirus systems may have impact on pest control and viral ecology, but certainly this work will advance our fundamental knowledge of insect endocrinology.

Some years ago the hypothesis was advanced that juvenile hormone regulation in some insects could be accomplished by a combined decrease in biosynthesis and an increase in degradation (Hammock, 1985). Although regulation of chemical mediators by degradation is common with neurotransmitters and neurohormones, variations in the rates of degradation have not been widely considered as significant with epithelial hormones.

In insects, which must effect major changes in hormone titer in short periods of time, it is not surprising that regulation of both biosynthesis and degradation should be employed. With juvenile hormone this possibility was certainly suggested in *Manduca sexta* when Weirich et al. (1973) reported a correlation between the ability of the hemolymph to hydrolyze the methyl ester of JH and the reduction of JH titer. Experimental support for this hypothesis resulted first from the demonstration that inhibition of this juvenile hormone esterase (JHE) with selective organophosphates gave developmental effects similar to the application of juvenile hormone (Sparks and Hammock, 1980). The case was strengthened when it was found that a novel group of highly selective "transition state mimics" of JHE which resembled JH could inhibit the enzyme and result in similar giant larvae (Hammock et al., 1984). This hypothetical role of JHE was further supported by the demonstration that inhibition of JHE blocked the complete clearance of JH when monitored by GLC-MS. This work demonstrated that very little JH was needed to block metamorphosis and provided an explanation for why reduced biosynthesis alone could not clear JH in preparation for pupation (Jones et al., 1989).

The alternate approach to testing this hypothesis of JHE involvement in the initiation of metamorphosis is to demonstrate anti-JH effects elicited by the precocious appearance of JHE. In this manuscript we summarize recent work from our laboratories on the biochemistry and molecular biology of JHE leading to such experiments. It is hoped that the results of this work will help to elucidate mechanisms of JH regulation in other systems. However, it must be cautioned that the regulatory mechanisms are likely to be very different in other species.

ISOLATION OF ENZYME AND MESSAGE

Since JHE is a low abundance protein present in a limited tissue source, the development of a highly efficient affinity purification system for the enzyme was critical. The trifluoromethylketone column resulting from this work proved very efficient for purifying the JHE from the hemolymph of a variety of lepidopterous larvae if the general esterases had been previously inhibited (Abdel-Aal and Hammock, 1986; Hammock et al., 1988). In fact this column appears generally applicable to the purification of esterases from many species.

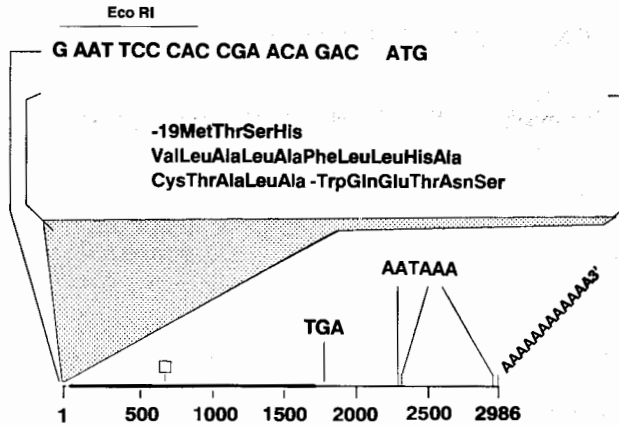


Fig. 1. Key characteristics of the sequence of a 2986 bp JHE message from *H. virescens*. Following the *EcoRI* site is a consensus sequence for an insect ribosome binding site (top). Following the first ATG there is a putative 19 amino acid leader sequence before a deduced N-terminal sequence starting with TrpGln which matches closely the sequence determined by Edman degradation. The coding region is shown by the bold line and the hypothetical catalytic serine by a square. Following the stop codon is long noncoding region shown by a light line with 3 polyadenylation sites. The numbers at the bottom refer to the bases of the cDNA.

The resulting pure proteins allowed the determination of N-terminal sequences and the development of antibodies for the JHE's from *Heliothis virescens*, *M. sexta*, and *Trichoplusia ni*. These probes were used to isolate three apparently full length messages from a fat body cDNA library of *H. virescens*.

The three clones (3hv1, 3hv16 and 3hv21) had similar sequences. As shown in Fig. 1 one of the clones (3hv21) had an insert of 2989 bp which approximates the length of the putative JHE message detected by Northern blot. The derived amino acid sequence from the 1714 bp open reading frame predicts a 563 amino acid 61 kDa protein which agrees well with SDS-PAGE analysis of the hemolymph protein (Hanzlik et al., 1989).

SEQUENCE ANALYSIS

Comparison of these JHE sequences with other known esterases may provide some insight into the catalytic mechanism of esterases. Over 20 percent homology was observed among JHE and several other esterases suggesting that these enzymes arise from a common ancestral enzyme. The region of greatest similarity lies near the N-terminal end of the protein (Fig. 2).

In the early days of X-ray analysis of protein crystals Blow and his associates developed an exciting hypothesis to explain the high reactivity of the catalytic serine of proteases based on proton transfer from serine through histidine to aspartic acid (Blow, 1976). With some adjustment this catalytic triad theory still seems applicable to protease action (Smith et al., 1989). Since esterases are known to have a similar catalytically active serine, it was not surprising that the triad was also assumed to exist in esterases. However,

sequence analysis of a variety of esterases indicated that either esterases have a radically different folding pattern than proteases or that a different catalytic system was involved. A single catalytically important serine is present in the Gly-X-Ser-X-Gly sequence seen in proteases, but if the later hypothesis is correct the catalytic triad is replaced by highly conserved regions corresponding to Asp 102 and His 57 of chymotrypsin. The acid motif of most esterases would accommodate either aspartic or glutamic acid acting as the final proton acceptor of a catalytic triad. However, JHE has only aspartic acid. The situation is more complex if one tries to find a basic amino acid substitute for the histidine 57. The Arg-Phe region of esterases corresponds to this His motif of proteases. Although Arg could orient and activate a water molecule involved in a proton transfer reaction, polarize the ester carbonyl, or even activate serine directly, it is difficult to envision how it could play an analogous role to that hypothesized for His in proteases (Hanzlik et al., 1989).

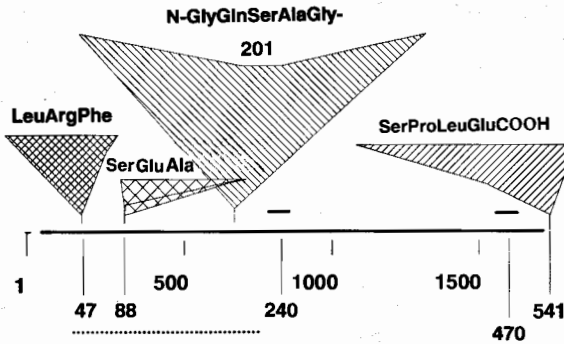


Fig. 2. Deduced amino acid sequences of putative catalytic motifs of the 1.7 kbp translated region of the JHE cDNA. In addition to the C-terminal sequence, three sequences which may correspond to the catalytic triad of serine proteases are shown. The two small solid lines show polar regions of acetylcholine esterase thought to be involved in substrate binding which are nonpolar in JHE. The dotted line indicates the region of greatest homology with the other serine esterases and thyroglobulin. The upper numbers indicate base number and the lower ones refer to amino acid number.

There are a variety of other highly conserved motifs in esterases which may be catalytically important. However, X-ray studies and site directed mutagenicity are needed to support hypotheses on the catalytic mechanism of esterases. It will be interesting to see if the highly conserved Arg-Phe region is catalytically important.

GENE REGULATION

The regulation of JHE appears complex. Its appearance in the hemolymph early in the last larval stadium appears to be under a different control system than its appearance during the prepupal period (Hammock, 1985). Further complexities exist when one considers the vastly different activities present in tissues and hemolymph in the pupa, its low apparently constitutive appearance in tissues in early instars and its brief appearance in the hemolymph during larval molts in *T. ni*. Our understanding of this regulation is at best superficial. However, an 851 bp *Bam*HI fragment from the 5' end of the *H. virescens* JHE clone has been used to detect both RNA and DNA from several species. Hopefully this and other probes will accelerate aspects of the above work on JHE regulation in a number of laboratories.

When this fragment was used to probe RNA isolated from larvae of *H. virescens* of different ages, a 3 kbp fragment was always found by Northern analysis. The message was not detected in third stadium larvae when JHE expression is very low, but was detected in the last larval stadium. On day 2 of this stadium, mRNA levels in the integument were 3 times higher than in the fat body. However, two days later when hemolymph activity of JHE reaches peak levels, the JHE mRNA detected in the fat body increased 9 times while

the levels in the epidermis decreased slightly. These data suggest that JHE can differentially regulated in tissues and that the majority of the JHE in peak hemolymph level comes from the fat body. The level of message and catalytic activity in both tissues could be dramatically increased by treatment of whole insects with the juvenoid epifenonane.

Since both message and protein levels (determined by Western blot) correlate with the catalytic activity of JHE, it appears that transcriptional control is very important in regulating JHE expression. However, it also seems clear that the ability of a tissue to hydrolyze JH is controlled by a variety of factors including the stability of the message and protein (Wroblewski et al., 1989).

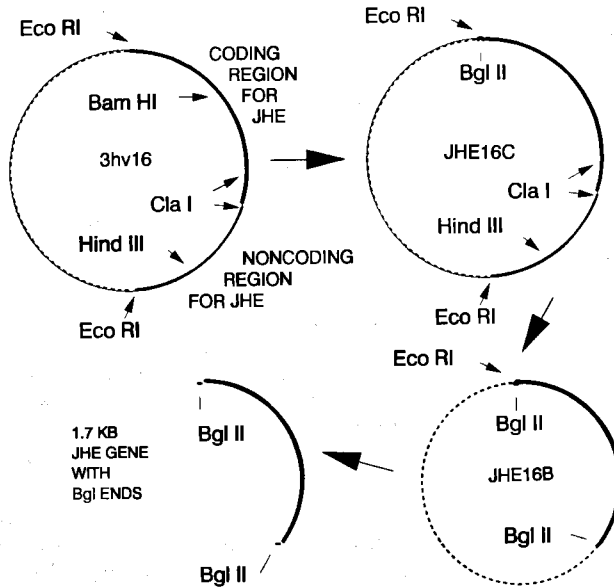


Fig. 3. Plasmids used in the isolation of 1.7 kbp coding fragment of the juvenile hormone esterase from *H. virescens*. The Bluescript plasmid is represented by the dotted line and the 3' noncoding and coding regions of the JHE gene by increasingly heavier lines. Plasmid 3hv16 is described by Hanzlik et al. (1989). Plasmid JHE16C was modified with *Bgl*II linkers flanking an *Eco*RI site at the 5' end of the gene. Plasmid JHE16B has the DNA from the *Cla*I site downstream from the natural stop codon through the *Cla*I site in the polylinker removed and replaced by a *Bgl*II linker.

BACULOVIRUS EXPRESSION OF JHE

The development of an expression system for JHE allows one to address a variety of problems. It obviously allows one to test if the isolated sequence is catalytically active as well as facilitating the production of large amounts of enzyme for biological and pharmacokinetic studies. Since the cell lines used are of lepidopterous origin, it is likely that expression will shed some light on the natural processes of post translational modification and transport of the enzyme. Finally, from a practical perspective, it may lead to the production of baculoviruses of improved characteristics. Toward these ends we are working on the expression of full and partial sequences of JHE in baculovirus systems from *Autographa californica* and from *Bombyx mori*.

Expression with NPV of *A. californica*

For expression in this system a transfer vector (pAcRP23) was used which had a *Bam*HI cloning site (Matsura et al., 1987). This presented some difficulty since the JHE plasmid used had an internal *Bam*HI site as discussed above. Thus, as shown in Fig. 4 the 3hv16 plasmid was linearized by partial digestion with *Eco*RI. The ends of the purified DNA were filled in with the Klenow fragment of *E. coli* DNA polymerase, *Bgl*II linkers

ded, and the DNA religated to give a 5' *Eco*RI site flanked by *Bgl*II sites. This plasmid (JHE16C) was grown and DNA again isolated. A partial digestion with *Cla*I removed most of the 3' noncoding region while retaining the natural stop codon and the full 1.7 kbp coding region. The above process of adding *Bgl*II linkers at the 3' end of the JHE gene was used to give plasmid JHE16B (Fig. 3). *Bgl*II digestion of this plasmid yielded the 1.7 kbp fragment which could be easily cloned into the *Bam*HI site of pAcRP23 to yield pAcRP23.JHE. This recombinant transfer vector was co-precipitated with wild type virus on to cells of *Spodoptera frugiperda* (IPLB Sf 21) and a recombinant virus isolated by repeated plaque purification. In the virus AcRP23.JHE the JHE gene is under the control of the polyhedrion promoter and the polyadenylation site of the polyhedrion gene is used. This results in high levels of expression very late in infection.

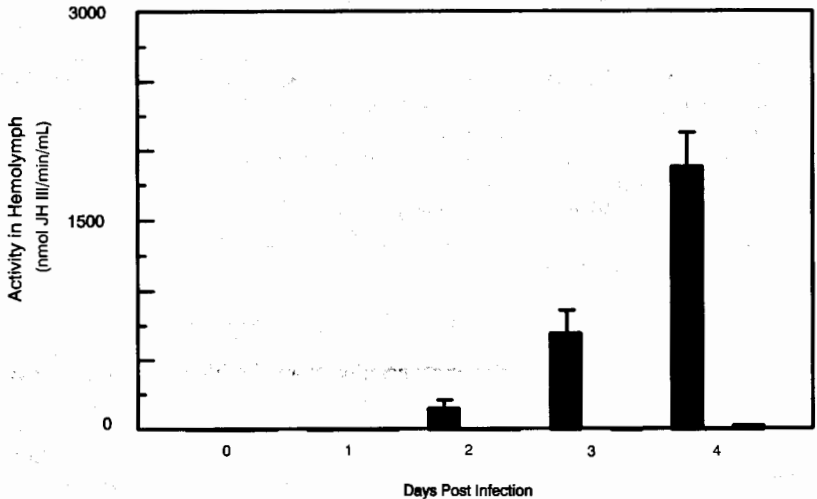


Fig. 4. Expression of JHE activity *in vivo* by a recombinant BmNPV virus. Larvae of *Bombyx mori* from the second day of the fifth instar were injected with 1.5×10^7 PFU's of the recombinant virus, 3hv1-1, that contained cDNA encoding JHE. Their hemolymph was then assayed for JH hydrolysis at one day intervals until death which occurred on the fifth day post infection (solid bar). The activities in the hemolymph of larvae injected with a recombinant that did not express JHE and sham injected larvae (right hatch) were also measured. No differences in morphology or weight gain between the control larvae and those injected with 3hv1-1 were noted. Data are the result of measurements from triplicate larvae. Data for larvae infected with the control recombinant virus were near zero and thus not apparent on the graph.

Since the ester of JH is so stable to most esterases, it was possible to detect high JHE activity even in crude transfections in the presence of high general carboxylesterase activity in fetal calf serum (FCS). It also was possible to monitor the JHE activity in agar associated with JHE producing plaques while *S. frugiperda* cells did not produce detectable JHE activity even when infected with several control viruses. Hydrolysis of JH by the crude culture medium from infected cells was found to be linearly dependent on protein concentration and time over a wide range of conditions. The expressed enzyme also was stable to repeated freeze thaw cycles, storage for several days at room temperature, and to a variety of denaturing reagents. This sensitive assay for JHE (Hammock and Sparks 1977) greatly simplifies the isolation of recombinant virus.

As expected from the leader sequence, the JHE is rapidly exported into the medium of infected cells. In all cases over 90 percent of the catalytic activity was found to be extracellular. Following infection in the presence of 5% FCS good production of JHE could be obtained in media lacking FCS and SDS-PAGE of the resulting media revealed JHE as the predominant protein as well as a major cellular protein. Depending on culture conditions up to 250 nmol/min of JH could be hydrolyzed per ml of culture media which is far higher than maximal rates obtained in the hemolymph of *H. virescens*.

When infected with the recombinant virus either orally or by injection, JHE activity is detected in the hemolymph of *T. ni* at levels far higher than normally present in early instar larvae. The levels are similar to those seen in normal early instar larvae at ecdysis. It is thought that the expression of JHE at this time results in a transient decrease in JH which reduces feeding behavior (Hanzlik and Hammock, 1988). However, the expressed levels are only about 10% of those normally seen in the last larval stadium of *T. ni* or *H. virescens*.

Expression with NPV of *B. mori*

For expression in the *B. mori* system, the full 3 kbp insert from the 3hv1 clone was used. Thus, it was possible for one of the 3 natural polyadenylation sites as well as the polyadenylation site of the polyhedron gene to be used. The JHE gene was removed from a Bluescript plasmid with *EcoRI* and cloned into an *EcoRI* site of the transfer vector pBE274. Cotransfection was carried out in a similar manner to that described above according to Maeda (1989). Expression *in vitro* was comparable to that observed in the *A. californica* system with the majority of the catalytic activity again detected in the media. However, expression *in vivo* with the *B. mori* system was very high. When fifth instar larvae of *B. mori* were injected with the recombinant virus, JHE activity could be detected as soon as two days post infection with activity increasing to protein levels corresponding to nearly 1 mg/ml in the hemolymph at 4 days post infection (Fig. 4). Death occurred with the recombinant virus as well as wild-type virus and a virus containing a defective JHE gene five days post infection. With both systems *in vivo* and *in vitro*, the protein purified by affinity chromatography was similar to that isolated from the hemolymph when analyzed by SDS-PAGE and Western blotting.

Biological JHE Effects

When JHE isolated by affinity chromatography from the blood of *M. sexta* or culture media of NPV infected cells of *S. frugiperda* was injected into third stadium larvae of *M. sexta*, the larvae turned black at the following molt in a dose dependent fashion. The effects of this injection of picomole amounts of JHE could be reversed by the application of a juvenoid resistant to ester hydrolysis. Thus, the natural and expressed enzymes have clear anti-juvenile hormone effects. However, we were unable to generate precocious pupae using the enzyme isolated from *M. sexta* possibly due to its short half life *in vivo* (Philpott and Hammock, 1989).

Infection of first stadium larvae of *T. ni* with AcRP23.JHE resulted in a reduction in feeding and weight gain. Although this reduction in feeding was not noticeable compared to uninfected control larvae or larvae infected with a virus expressing the gp 120 coat protein of HIV until 3-4 days post infection, the difference in size of the larvae by day 5 was dramatic. The size of larvae fed AcRP23.JHE, but not control viruses, was significantly increased by application of juvenoids. In contrast, no morphological changes have been observed in larvae of *B. mori* similarly infected with the recombinant NPV.

From the perspective of practical application, the lack of significant *in vivo* effects in *B. mori* are disappointing, but the effects in *T. ni* are very encouraging. Based on the biology of JHE it is likely that the insect has a mechanism which allows the rapid turn over of the enzyme. This may explain why *in vivo* expression of a JHE from a closely related species is so much lower than *in vitro* expression. The fact that low apparent expression of JHE under the control of a very late promoter can apparently overcome biosynthesis of JH and result in a biological effect indicates that higher levels of expression under an earlier promoter could improve the efficacy of viral insecticides.

The results from the above expression systems confirm that the isolated cDNA clones from *H. virescens* do code for JHE, that JHE may be a valuable reporter enzyme for studying promoter activity, and that expressed JHE can act as a biochemical anti-JH. Certainly the very sensitive catalytic assay for JHE coupled with sensitive immunochemical and hybridization assays will simplify detection of insects infected with the recombinant virus in ecological studies. It also is hoped that several approaches can be taken to increase the biological activity of the engineered viruses. Such work could involve the alteration of the DNA coding for JHE such that the message and/or expressed JHE protein is resistant to degradation in target insects as well as the examination of different viral promoter systems. However, the greatest value of the sequence for JHE will be as a probe in elucidating fundamental mechanisms of developmental regulation.

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