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AFFINITY PURIFICATION AND CHARACTERISTICS OF JUVENILE HORMONE ESTERASE FROM LEPIDOPTERA[1]

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ABSTRACT At several times during the development of lepidopterous larvae, the methyl ester of juvenile hormone is hydrolyzed by a highly specific group of esterases known as juvenile hormone esterase (JHE). Studies with inhibitors have demonstrated that JHE activity is essential for normal development. These inhibitors have included both organophosphates and 3-substituted thiotrifluoropropanones. The later compounds appear to act as "transition state mimic inhibitors" of JHE and yield slow tight binding kinetics. JHE is extraordinarily stable to extremes of pH, redox conditions and organic solvents. The enzyme also can be purified from a variety of species by affinity chromatography using the above transition state ligands. Experimental evidence indicates that the enzyme's catalytic site is involved in binding to the column.

INTRODUCTION

The elucidation of the mechanism by which larval insects initiate metamorphosis into pupae and then into adults remains one of the most exciting mysteries in developmental biology. Among those insects studied in the order Lepidoptera, a

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lowering of the titer of juvenile hormone (JH) and a subsequent release of ecdysone in response to the prothoracicotropic hormone certainly are key endocrine events in metamorphosis. The role of JH in this process certainly is more complex than was suggested by the "high-low-no" hypothesis (1). For instance JH has been detected in a variety of insects immediately before pupation. In Manduca sexta this burst of JH appears to retard precocious development of adult like structures (2) while in Trichoplusia ni the prepupal burst of JH is essential for successful pupation (3). However, the basic arguments summarized by Gilbert (1), that a profound reduction in JH titer is associated with the initiation of metamorphosis and that JH must be absent for pupation to occur, still hold.

It is generally accepted in endocrinology that regulation of hormone titers occurs by modulation of biosynthesis against a rather constant background of catabolism. Certainly a reduction in the rate of JH biosynthesis is a key factor in the decline of the hormone in the early part of the last larval instar. However, Weirich et. al., (4) reported that radioactive JH was metabolized to the corresponding JH acid by diluted hemolymph from M. sexta. Whether this JH esterase (JHE) activity was simply associated with the decline in JH or whether it played an active role in the decline remained in question.

The most direct answer to this question was provided by treating larvae of T. ni with a potent inhibitor of JHE and observing that the insects remained in the feeding stage and delayed their pupation (5). This experiment has been repeated with various insect species and JHE inhibitors from several different chemical classes (6-8). Several arguments support the presence of this regulatory system. First, the insect must reduce its JH titer dramatically over a very short period, thus a dual system of reducing biosynthesis while increasing catabolism would be of survival benefit. The concept that regulation of hormone titers was entirely by biosynthesis was developed in rather long lived species where such rapid changes in hormone titer were not critical. Possibly a better vertebrate system for comparison with larval Lepidoptera would be the nervous system where rapid changes are needed and where catabolism and reuptake are as if not more important than biosynthesis and release.

Second, JH is a very lipophilic material and can be anticipated to partition into lipophilic depots making precise regulation over a brief time scale difficult. To address this problem the insect has evolved a JH carrier system which helps to keep the hormone in solution (9). However, both the

lipophilic nature of JH and the presence of a carrier protein for the hormone reduce turnover making it difficult for the insect to effect dramatic reductions in JH titer over a short period of time. Thus, an enzyme system which can extract the hormone by mass action from these niches is critical for JH reduction. One can further argue that when the JHE is present, the carrier protein speeds degradation of JH by keeping it in solution, while when enzymes with a high affinity for JH are absent, that the carrier protein prolongs the life of JH (10).

Third, there is evidence that during wandering when the titer of JH is reduced dramatically, that the activity of the corpora allata is reduced but not eliminated. Since the JH titers in T. ni larvae just before pupation were the highest detected during the last two instars of larval life, the corpora allata must increase their biosynthetic capability again in the prepupa. Thus, it is reasonable to assume that it is efficient for the insect to reduce JH production but not to dismantle its biosynthetic machinery for the hormone. Since even trace amounts of JH will disrupt development during the change of commitment from larva to pupa, it seems critical that the insect degrade existing hormone as well as small amounts of JH that continue to be produced.

Thus, presence of JHE is critical for the normal metamorphosis of lepidopterous larvae. In addition, its appearance in high levels in the hemolymph is one of the earliest events in the developmental sequence leading to pupation. The molecular probes developed to investigate its appearance may lead to the discovery of still earlier events in development. Preliminary studies indicate that its initial appearance is under the control of factor(s) from the brain and subesophageal ganglion with juvenile hormone playing a minor role. However, its subsequent appearance clearly is influenced directly by the prewandering peak of JH in M. sexta and T. ni (11-13). Aspects of JH degradation and the regulation of JHE have been covered in previous reviews (9,10,14-17). This manuscript will describe the biochemical properties of the enzyme itself.

INHIBITION OF JHE

It was early noted that O,O-di-isopropyl phosphofluoridate (DFP) was a poor inhibitor of the enzymes metabolizing JH in M. sexta while it is a powerful inhibitor of most serine esterases and proteases (18). It subsequently has proven to be very useful in selective inhibition of other

esterases in insect hemolymph while preserving JHE. Yet, it is important that this tool is not overused. Hammock (10) discussed various definitions of JHE with the simplest being any esterase which hydrolyzes JH. DFP appears to be a powerful tool in some insects for distinguishing the esterase with a low K_M for JH from other esterases probably due to the steric hindrance of the isopropyl groups. However, this apparent selective inhibition cannot be applied without testing each system individually. Older samples of DFP were almost inactive as inhibitors of JHE while being extraordinarily powerful inhibitors of general carboxylesterases of insect hemolymph and mammalian liver. Some recent samples of DFP, however, seem to contain trace impurities which inhibit JHE (Roe, unpublished).

DFP has proven useful in inhibiting esterases not involved in JH hydrolysis in Lepidoptera, while O-ethyl S-phenyl phosphoramidothioate (EPPAT) has proven to be a powerful, irreversible inhibitor of JHE in vivo and in vitro (5,19-21). Other, commercially available compounds such as paraoxon are very useful for inhibiting JHE in vitro, but their high toxicity limits their use in vivo (5).

Two series of reversible inhibitors that have proven exceptionally useful include substituted trifluoroketones ($RC(O)CF_3$) and 3-substituted thio-1,1,1-trifluoro-2-propanones ($RSCH_2C(O)CF_3$). The former compounds appear to be classical reversible inhibitors with K_i's as low as 3.2×10^{-9}M (22). It is interesting that the structure activity relationships among these compounds suggest that the properties needed for binding to JHE are in some ways similar to the properties of JH which the enzyme recognizes. The trifluoroketones appear to act as "transition state analogs" yet the compounds appeared to be inactive in in vivo bioassays designed to detect their ability to dramatically reduce the rate of JH catabolism.

The more potent thiopropanones yielded complex kinetics when they were tested as inhibitors of JHE (7,8,23-25). They can be classified as reversible inhibitors since it is likely that the compound which dissociates from the enzyme has the same chemical structure as the compound which bound to the enzyme. However, as is common with many powerful, reversible inhibitors, these compounds appeared to give slow tight binding kinetics. Possibly the slow dissociation of the inhibitor from the catalytic site of JHE as well as its high affinity for the site allowed one to detect JH-like effects when the compounds were applied in vivo. As will be discussed later, the affinity of these compounds for JHE and their unique kinetic behavior have proven to be very useful.

TABLE 1
KINETIC PARAMETERS OF JHE AND JH BINDING PROTEIN(S) FROM T. ni USING JH II AS SUBSTRATE OR LIGAND

Kinetic Parameter	JHE	JH Binding Protein
Molar concentration in the plasma at near maximum levels	1.49×10^{-6}	8.1×10^{-6}
k_{cat}	31.8 min^{-1}	Not Applicable
K_m(M)	7.06×10^{-8}	1.75×10^{-7}
V_{max} nmoles/min/ml	65.0	Not Applicable
k_d min^{-1}	Not Applicable	9.32×10^{-2}
$k_a M^{-1} min^{-1}$	Not Applicable	5.33×10^{5}
k_{cat}/K_M $M^{-1}min^{-1}$	4.50×10^{8}	Not Applicable
	$[k_{cat}/K_M] \times E_t$	$k_a \times$ [B.P.]
	$6.72 \times 10^{2} (min^{-1})$	$4.32 (min^{-1})$
$t_{0.5}$ Sec.	0.062 (For hydrolysis)	9.62 (For association)
Relative $t_{0.5}$	1.00	155

KINETIC CONSTANTS OF JHE

A variety of factors, including the low solubility of JH, have made it difficult to obtain clean estimates of the kinetic constants of JHE in insect hemolymph. Recently by using the almost stoichiometric binding of some trifluoromethylketone inhibitors of JHE, it has been possible to titrate the catalytic sites of JHE in crude hemolymph using Ackerman-Potter plots (23). The concentration of JHE in the hemolymph determined by this method is surprisingly close to that estimated by affinity purification of the enzyme (25,27). Careful kinetic studies on both the crude and the affinity purified enzyme have given rise to kinetic constants such as those shown in Table 1.

In most physiological systems one assumes that the substrate concentration approaches the K_M of the enzyme which metabolizes it. However, JHE appears to function as a scavenger enzyme and the titer of JH II in T. ni is far below the K_M of JHE. Thus, the ratio of k_{cat} to K_M is an appropriate kinetic parameter to use in estimating the capacity of the enzyme to hydrolyze JH under in vivo conditions. Since JH II is by far the major JH in the ultimate larval instar of T. ni, it is the only substrate treated here. The k_{cat}/K_M ratio of JHE indicates that degradation of the hormone by the enzyme approaches the diffusion controlled encounter of enzyme and substrate.

It appears clear from previous studies that there is adequate JH binding protein in the hemolymph to complex the majority of the JH present. The relatively low K_M of JHE and its high turnover indicate that it is likely to hydrolyze JH molecules as soon as they are released by the JH binding protein in the hemolymph. A numerical argument for this can be made by noting that the $t_{0.5}$ for hydrolysis of JH II by JHE is approximately 150 times faster than the $t_{0.5}$ for association of the JH II with the hemolymph binding protein (Table 1). These data provide support for the argument presented in the introduction for the dual role of the JH binding protein in both stabilizing JH when the JHE is at low titers and enhancing clearance when JHE is present. These data also indicate that insect control strategies based upon inhibition of JHE would be very unlikely to succeed. However, it is likely that even limited expression of JHE at inappropriate times during development could overpower the biosynthetic capacity of the corpora allata to produce JH.

STABILITY OF JHE

While examining the influence of a variety of reagents on JHE activity, it was noted that JH is exceptionally stable to a variety of treatments which denature many enzymes. These treatments include high levels of oxidants, inorganic ions, many classical esterase inhibitors, and extremes of pH. For instance when JHE from T. ni was incubated in H_2O_2 for 10 minutes at 30.C, it retained 94 percent of its catalytic activity. As reported earlier by several workers, JHE is catalytically active over a wide range of pH's (FIG. 1). This figure also shows that the enzyme is very stable to extremes of pH's for extended periods when analyzed at pH 7.4. It was observed that some organic solvents dramatically increased the activity of JHE of M. sexta both in the crude hemolymph and as pure enzyme. Such activation was noted earlier for other carboxylesterases by a variety of workers including Barker and Jencks (26) who attributed the behavior to a modifier site in porcine hepatic esterases. However, the activation of JHE from M. sexta was much higher than that observed in vertebrate systems with activation as high as 1200 percent with acetone at a concentration of 2 M. Activation was also found with low concentrations of ethanol with 147 percent of control enzyme activity found with an ethanol concentration of 0.17 M (1 percent ethanol). This certainly indicates an effect at solvent concentrations commonly used in enzyme assays.

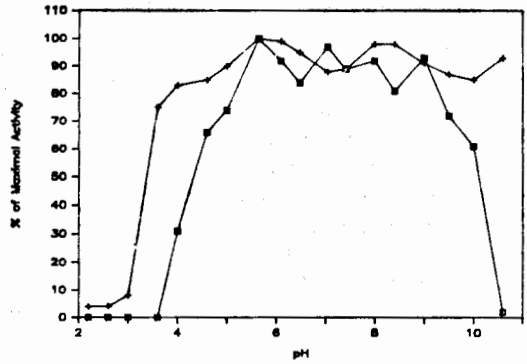

FIGURE 1. Relative activity of JHE partially purified from prepupal homogenates of T. ni when incubated with JH III at different pH's (■) or held at 4.C at varying pH's then diluted with sodium phosphate buffer to pH 7.4 for analysis (+).

It is possible that such a regulator site could be of biological significance in influencing the activity of JHE *in vivo*. However, the JHE's from other Lepidoptera examined do not seem to be influenced as dramatically by the presence of solvents as does the enzyme from *M. sexta*.

AFFINITY PURIFICATION OF JHE

Because of its interesting catalytic properties and its apparent biological role there has been interest in purifying JHE. However, the low concentration of the enzyme in the hemolymph and the difficulty in obtaining large amounts of biological material has made classical purifications very difficult (10). Since some trifluoropropanones appeared to bind to JHE with a high degree of selectivity, these compounds were used as affinity ligands in an effort to purify the enzyme from several species.

$$HS(CH_2)_4SH + BrCH_2C(O)CF_3 \rightarrow HS(CH_2)_4SCH_2C(O)CF_3$$

FIGURE 2. Synthesis of a ligand for the affinity purification of JHE.

Several ligands have been examined, but the simplest results from the reaction of 1,4-butane dithiol with 3-bromo-1,1,1-trifluoroacetone as shown in FIGURE 2. The resulting thiol is reacted in turn with epoxy activated Sepharose. When 10 ml of diluted hemolymph was passed through 25 microliters of this gel no decrease in protein content was noted in the effluent but over 99 percent of the JHE activity was absent. Since JHE is such a minor protein that it cannot be seen as a discrete band on a SDS-PAGE gel of crude hemolymph, proof that the enzyme was actually bound to the column was not straight forward. Mixing of the effluent with inhibited enzyme indicated that the column was not bleeding an inhibitory substance. However, removal of the JHE activity bound to the column proved to be a difficult task.

Detergent, ionic, pH and other gradients alone or in combination failed to remove significant levels of JHE activity or protein. Even when gradients containing powerful inhibitors

of JHE were run through the column, the bound esterase was not recovered. The solution to this dilemma came from recalling that the thio trifluoropropanones were slow tight binding inhibitors. Thus, when an inhibitor solution was allowed to incubate with the affinity gel for an extended period, excellent and sometimes quantitative recovery of JHE was obtained (25,27).

Recovery of enzyme activity also was difficult since very powerful inhibitors such as 3-octylthio-1,1,1-trifluoro-2-propanone (OTFP) were used to elute the enzyme from the column. Fortunately, JHE is very stable in both the crude and purified states and long dialysis times were successful in removing the inhibitor and in recovering the catalytic activity. As shown in FIGURE 3, recovery of enzyme activity is more rapid at acidic pH's. This increased rate of recovery probably results from a combination of two reasons. First, reduced catalytic activity of JHE was noted at low pH (FIG. 1), and this may translate to reduced binding of the enzyme to the inhibitor.

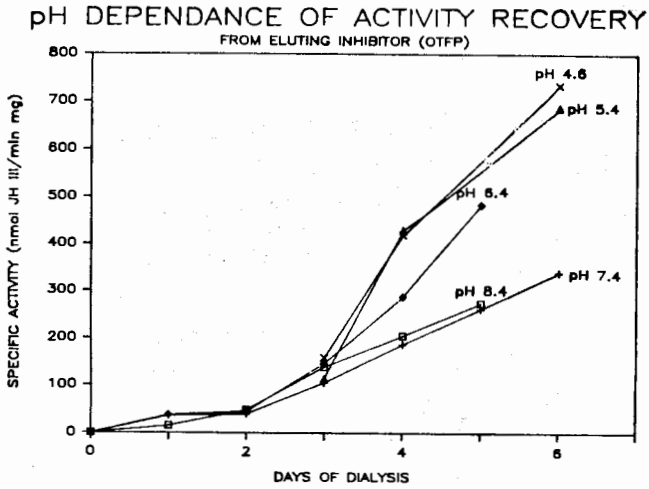

FIGURE 3. Plot of the pH dependant rate of recovery of JHE activity from T. ni during dialysis of the the enzyme eluted from the affinity column by OTFP. Equal amounts of eluted enzyme containing 200 μg/ml BSA were dialyzed against buffer containing 0.2M sodium phosphate or acetate buffers with 0.01% phenyl thiourea, 5% sucrose and 0.02% sodium azide at room temperature. All samples were assayed at pH 7.4. Full recovery of this enzyme takes approximately 2 weeks.

Second, acidic conditions greatly accelerate the chemical formation and decomposition of hemiketals which could accelerate dissociation of the enzyme and inhibitor. In fact this experiment provides circumstantial evidence that the enzyme inhibitor complex resembles a hemiketal.

As mentioned above, JHE is thought to bind to the affinity column by interaction of the trifluoroketone with the catalytic site. FIGURE 4 provides direct evidence for this hypothesis by showing that inhibitors of JHE block its binding to the column. These data are from whole body homogenates of day 2 last instar larvae of T. ni which contain several proteins binding to the affinity column. The inhibitors were $2x10^{-4}$M DFP, $1x10^{-5}$M EPPAT, and $1x10^{-5}$M OTFP. Enzyme activity was monitored during the batch loading procedure and showed that little JHE activity was lost from the control and DFP treated homogenate prior to addition of the affinity gel. However, the activity dropped sharply once the gel was added. In the homogenates treated with OTFP and EPPAT, no JHE activity was present upon addition of the gel. Following loading, the

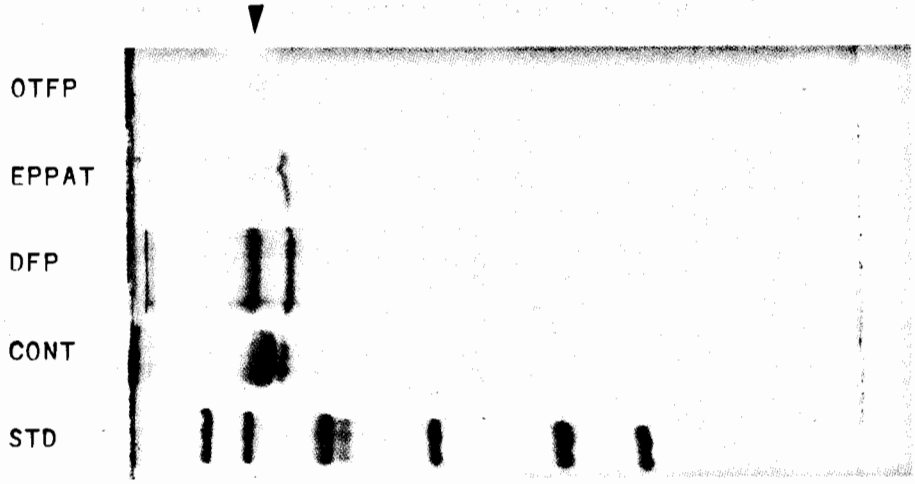

FIGURE 4. SDS-PAGE gel of proteins recovered from affinity purification of homogenates of T. ni treated with various inhibitors. A more complete washing of the gel resulted in a 1 step purification of JHE migrating with an estimated Mr of 65,000 shown by an arrow. Standards of molecular weights 92.5, 66.2, 45, 31, 25, and 14.5 KD are in the left (bottom) lane of the gel.

gel was washed and eluted according to standard procedures. As shown in FIGURE 4, no JHE was detected in the samples treated with EPPAT or OTFP while both protein and activity were recovered from the control and DFP treated gels.

The trifluoroketone affinity column has proven to be very useful for a variety of purposes. For instance, it is directly applicable to the purification of JHE from a variety of species as shown in FIGURE 5.

Although classical purification procedures for JHE are conceptually feasible, small biomass makes it difficult to purify sufficient quantities of a low abundance protein for subsequent studies. The high yield of the affinity chromatography procedure has made it possible to purify sufficient material to radiolabel the catalytic site of JHE from the above species, raise antibodies and obtain sequence formation (25,27). The latter data indicate the similarity among JHE isozymes in that the N-terminal sequence for the major component of JHE from H. virescens is TRP-GLN-R while the minor component has two additional amino acids giving a sequence of SER-ALA-TRP-GLN-R. The affinity column has also made it possible to compare JHE isolated from different tissues and from different stages of larvae.

The chemical and molecular probes developed for JHE should be of great value in expanding our understanding of this regulatory enzyme. Hopefully, this technology can be applied to biologically interesting enzymes in other systems.

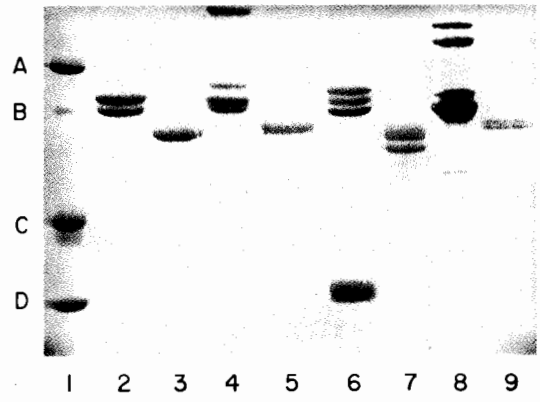

FIGURE 5. SDS-PAGE of the crude hemolymph (lanes 2,4,6,8) and affinity purified JHE (3,5,7,9) from M. sexta, H. virescens, B. mori, and H. zea, respectively. Protein andards from 92.5 to 31KD are shown in lane 1. Adapted from Abdel-Aal and Hammock (26).

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