Juvenile hormone (JH) esterase: why are you so JH specific?

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Abstract

Juvenile hormone esterases (JHEs) from six insects belonging to three orders (Lepidoptera, Coleoptera, and Diptera) were compared in terms of their deduced amino acid sequence and biochemical properties. The four lepidopteran JHEs showed from 52% to 59% identity to each other and about 30% identity to the coleopteran and dipteran JHEs. The JHE of *Manduca sexta* was remarkably resistant to the addition of organic co-solvents and detergent; in some cases, it demonstrated significant activation of activity. Trifluoromethylketone (TFK) inhibitors with chain lengths of 8, 10 or 12 carbons were highly effective against both lepidopteran and coleopteran JHEs. The coleopteran JHE remained sensitive to TFK inhibitors with a chain length of 6 carbons, whereas the lepidopteran JHEs were significantly less sensitive. When the chain was altered to a phenethyl moiety, the coleopteran JHE remained moderately sensitive, while the lepidopteran JHEs were much less sensitive. The lepidopteran and coleopteran JHEs did not show dramatic differences in specificity to α-naphthyl and ρ-nitrophenyl substrates. However, as the chain length of the α-naphthyl substrates increased from propionate to caprylate, there was a trend towards reduced activity. The JHE of *M. sexta* was crystallized and the properties of the crystal suggest a high-resolution structure will follow.

1. Introduction

Using a keen sense of scientific intuition and steady hands for surgical techniques, Wigglesworth (1935, 1936) was the first to identify "juvenile hormone" (JH) as a factor produced by the *corpora allata* glands that prevented juvenile insects from molting into adults. About 30 years later, Röller et al. (1967) were the first to deduce the chemical structure of juvenile hormone (JH I). Subsequently, Meyer et al. (1970) confirmed the structure of JH I and identified the structure of a second juvenile hormone (JH II). This and other historical aspects of the discovery and characterization of JHs are elegantly reviewed by Gilbert et al. (2000). Perhaps the most wonderful and interesting aspect of JH is the exceptionally diverse range of functionality that JH and/or JH metabolites have on the insect life cycle including roles in development, metamorphosis, reproduction, diapause, migration, polyphenism, and metabolism (reviewed in Roe and Venkatesh, 1990; Riddiford, 1994; de Kort and Granger, 1996; Gilbert et al., 2000). These diverse functionalities suggest that not only are there numerous target sites for JH, but also that its biosynthesis, transport, and degradation must be carefully regulated. Slade and Zibitt (1971) demonstrated that JH was degraded by both ester cleavage and epoxide hydration and that the relative rates of degradation varied with the species and stage of the insect used. As usual, the Gilbert laboratory was one of the first to contribute to the juvenile hormone esterase (JHE) field (Whitmore et al., 1972). Much of the early work on the interaction

of hemolymph JH binding proteins and JHE came from the laboratories of John Law and Larry Gilbert (Whitmore and Gilbert, 1972; Kramer et al., 1974; Goodman et al., 1978).

Our laboratory has advanced the hypothesis that regulation of JH is due not only to changes in the rates of its biosynthesis, but also in the rates of its degradation. This has been most clearly shown in lepidopteran larvae where inhibition of JHE reduces the rate of JH degradation and leads to abnormally large larvae and delayed pupation (Sparks and Hammock, 1980; Hammock et al., 1990). Two pathways for the degradation of JH have been intensively studied in insects (reviewed in Hammock, 1985; Roe and Venkatesh, 1990; de Kort and Granger, 1996; Gilbert et al., 2000). One involves the hydrolysis of the methyl ester moiety at one end of the JH molecule by a soluble esterase resulting in the conversion of the methyl ester into a carboxylic acid. The other involves hydrolysis of the epoxide moiety at the other end of the JH molecule by a microsomal epoxide hydrolase resulting in a diol. Both the esterase and hydrolase are members of the α/β-hydrolase fold family and have homologous mechanisms, although they are widely separated in evolutionary history. Our laboratory (Hammock, 1985) has proposed a possible definition of a JH-selective esterase by both biological and biochemical criteria. Biologically, JHE should have an activity that is correlated with a decline in the titer of JH, and the enzyme should be essential for the clearance of JH from the insect's body. Also, the JHE enzyme should have a low apparent K_m for the substrate JH, and therefore hydrolyze JH with a high k_{cat}/K_m ratio. Further evidence for supporting the role of an enzyme as a JH-selective esterase could be obtained by specifically reducing (with inhibitors or RNAi) or increasing (by recombinant means or injection) the JH-selective enzyme activity. Of course, we must remember that the term JH-selective esterase may be misleading in that the enzyme may actually be involved in other processes.

2. Inhibitors of JHE

An exceptionally useful tool to study the role of JHE in vivo has been the use of chemical inhibitors containing a trifluoromethylketone (TFK) (Fig. 1). These com-
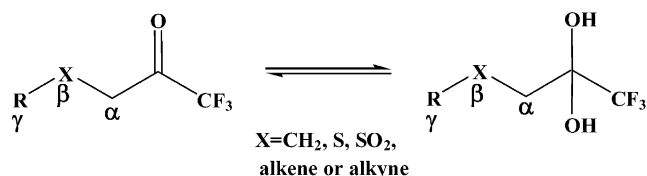
pounds are the most potent inhibitors of JHE identified to date. TFK inhibitors have also proven to be important for the purification of the JHE enzyme, determining its physiological role, and eliminating metabolism so other aspects of its physiology can be determined. A second class of useful inhibitor includes the phosphoramidothiolate, *O*-ethyl-*S*-phenyl phosphoramidothiolate (EPPAT). By application of EPPAT to larvae of *Trichoplusia ni* (Lepidoptera), Sparks and Hammock (1980) were able to show that JHE inhibition extends the feeding stage and delays pupation. Hammock et al. (1982, 1984) first reported that substituted thiotrifluoropropanones such as 3-octylthio-1,1,1-trifluoro-2-propanone (OTFP) are more potent inhibitors of JHE than their alkyl analogs. Although OTFP is less persistent (requiring greater amounts of application) than EPPAT by in vivo application to larvae of *T. ni*, it is as effective at delaying pupation and less toxic (Hammock et al., 1984). The most potent TFK inhibitors contain a long chain aliphatic tail that is thought to mimic the backbone of JH. The electron withdrawing trifluoromethyl group increases the electrophilicity of the carbonyl carbon, thereby increasing its susceptibility to nucleophilic attack by the catalytic serine. It has been postulated that TFKs act as transition state analogs (TSA) due to their mimicking of the tetrahedral transition state of the enzyme/substrate complex (Hammock et al., 1982; Hammock et al., 1984). Prestwich et al. (1984) tested further mimics of the terpenoid backbone of JH by adding alkyl substitutions and varying degrees of unsaturation in the hydrocarbon backbone. Their experiments indicated that in terms of mimicry, chain length and a methyl group at the alpha position had the greatest effects on the potency or selectivity of the inhibitor in the hemolymph of *T. ni*. The effects of JH mimicry were also extensively examined by the Roe laboratory (Linderman et al., 1987; Linderman et al., 1989; Roe and Venkatesh, 1990) who came to a similar conclusion. More recently, Roe et al. (1997) synthesized another potent inhibitor, 1-octyl (1-(3,3,3,-trifluoropropan-2,2-dihydroxy))-sulfone (OTFPdOH-sulfone) that is highly specific for JHE and significantly more stable in vivo in comparison to OTFP. By topical application of OTFPdOH-sulfone, they showed a greater than 90% reduction in plasma JHE activity during the feeding stage of larvae of *T. ni* that resulted in a significant delay in pupation. Topical application of OTFPdOH-sulfone in adults of *T. ni* results in a significant increase in egg oviposition. Additionally, they found that the extent of hydration (Fig. 1) of the inhibitor is an important factor for JHE inhibition (Roe et al., 1997). This chemistry has been further explored by Wheelock et al. (2001, 2002) who showed using ab initio calculations that the hydration state of the ketone is affected by its surrounding chemical moieties and directly related to inhibitor potency. We have reported that inhibitors that favor the hydrated form of the inhibi-

Fig. 1. TFK structures showing hydration from the ketone to the *gem*-diol. The various R moieties are shown in Tables 1 and 2. Greek letters refer to atom position relative to the carbonyl carbon.

tor show increased potency. However, if the inhibitor is "too hydrated" (i.e., the ketone-hydrate equilibrium is shifted too far towards the hydrate, see Fig. 1), inhibitor potency is reduced possibly due to a reduction in the concentration of the ketone (assuming that the ketone is the active form of the inhibitor rather than the *gem*-diol) (Wheelock et al., 2002).

Following the development of the TFK containing chemicals as potent and selective inhibitors of JHE, these inhibitors have also been attached to Sepharose and used as ligands for the affinity purification of JHE and other enzymes (Abdel-Aal and Hammock, 1986). The efficiency of this strategy has been greatly improved by first "removing" general esterases from the protein preparation using a general inhibitor of carboxylesterases such as diisopropyl phosphofluoridate (DFP) (Hanzlik and Hammock, 1987; Hinton and Hammock, 2001). This subtractive purification strategy is possible because the JHE of some species is relatively insensitive to DFP. Furthermore, the JHEs obtained by this strategy are of sufficient purity and concentration for downstream uses such as the generation of antibodies and peptide sequencing. Peptide sequence data have in turn been used for the molecular cloning of their corresponding cDNAs by various methods including antibody screening of cDNA libraries from *Heliothis virescens* (Hanzlik et al., 1989) and degenerate PCR cloning from *Manduca sexta* (Hinton and Hammock, 2001), *Tenebrio molitor* (Thomas et al., 2000; Hinton and Hammock, 2003b), *Bombyx mori* (Hirai et al., 2002), and *Lymantria dispar* (Nussbaumer, unpublished).

3. Catalytic mechanism

The general catalytic mechanism of JHE is certain to be very similar to that of other lipases and esterases. However, we do not know the basis for JHE's exceptional selectivity for the JH structure, nor how it can efficiently hydrolyze a chemically stable conjugated ester. A crystal structure for JHE will be critical for the direct understanding of these and other questions regarding its biology and biochemistry. The baculovirus expression vector system (BEVS) has been used to express large quantities of authentic and mutated JHE protein for biochemical study including crystallization studies. Recombinant baculoviruses expressing JHE have also been used as tools for hypothesis testing with regard to the biological activity of JHE within the insect host (see van Meer et al., 2000) and as virus-based biological pesticides (reviewed in Inceoglu et al., 2001). BEVS has been used to express JHE from at least five different insect species (*H. virescens* (Hammock et al., 1990; Bonning et al., 1992), *Choristoneura fumiferana* (Feng et al., 1999), *B. mori* (Hirai et al., 2002), *M. sexta* (Hinton and Hammock, 2003a), and *T. molitor* (Hinton

and Hammock, 2003b)). Following the cloning and expression of the JHE from *H. virescens* (Hanzlik et al., 1989; Hammock et al., 1990), this and other laboratories have made several attempts to crystallize this enzyme. Unfortunately, the heavy glycosylation of the JHE of *H. virescens* precluded crystallization. Enzymatic removal of the sugar residues does not affect JHE activity (Ichinose et al., 1992), however, this process is prohibitively expensive for the generation of sufficient material for crystallization. Expression in *E. coli*, chemical inhibition of glycosylation, and removal of the putative glycosylation sites by site-directed mutagenesis all result in poor expression of the enzyme. Thus, having initially failed to crystallize the JHE from *H. virescens*, we developed a homology-based molecular model for this enzyme (Thomas et al., 1999). One insight from this model is that JHE is identical to other α/β-hydrolase fold enzymes in that it contains a catalytic triad made up of a nucleophile (Ser-203), a base (His-448), and an acid (Glu-334). We have previously demonstrated the involvement of these amino acid residues in JH catalysis by site-directed mutagenesis (Ward et al., 1992). Additionally, during comparison studies of our homology-based molecular model of JHE with a number of other esterases, a second serine (Ser-229) was found to be absolutely conserved in all of the known esterase/lipase sequences and structures supporting a hypothesis that esterases have a common catalytic tetrad rather than a triad (Thomas et al., 1999).

Although our initial attempts at crystallization with the JHE of *H. virescens* were unsuccessful, a breakthrough came with the purification, cloning and expression of the JHE enzyme from *M. sexta* (Hinton and Hammock, 2001, 2003a). The JHE of *M. sexta* is not glycosylated and attempts at crystallization yield crystals complexed with OTFP (Fig. 2A) that diffract to 2.8 Å in resolution (Fig. 2B), with unit cell dimensions of $a = 97.22$ Å, $b = 97.22$ Å, $c = 165.13$ Å, and $\alpha = \beta = \gamma = 90°$. Space group determinations are ambiguous, with $P4_12_12$ and $P4_32_12$, the likely space groups based on systematic absences in the crystallographic data. Attempts at determining molecular replacement solution were made using the EPMR software (Kissinger et al., 1999),
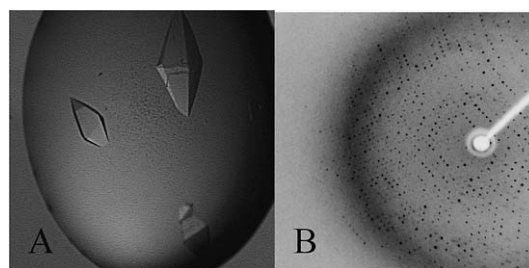

Fig. 2. (A) Crystals of the JHE of *M. sexta* complexed with the inhibitor OTFP. (B) Diffraction pattern of the JHE of *M. sexta* at a resolution of 2.8 Å.

with acetylcholine esterase from *Torpedo californica* (31% identity, 41% homology) (Raves et al., 1997). This method gave clear solutions in the $P4_12_12$ space group but not in the $P4_32_12$ space group, thus indicating that $P4_12_12$ is the correct space group. To date, approximately 75% of the structure has been modeled, and plausible electron density attributable to the OTFP inhibitor is visible.

4. Comparative analysis of JHE from different insect orders

In comparison to "general" esterases, JHEs are unique in terms of their selectivity and low K_m for JH (Peter, 1981; Wing et al., 1984; Campbell et al., 1998). This low K_m allows JHE to hydrolyze the JH substrate at far lower concentrations than other esterases. Additionally, many general esterases apparently cannot degrade JH. As described above, a high-resolution crystal structure of JHE should provide insight into the specific mechanism that imparts this unique specificity. Analysis of the biochemical properties of JHE enzymes from different insect orders and comparison of these properties with conservations in amino acid sequences should also provide insight into the mechanisms of specificity. In this light, we would like to present the following comparative analysis as a case study in the analysis of sequence-based functional analysis of the JHE protein. The newest JHE to be completely sequenced and expressed as a recombinant protein is the JHE from *T. molitor*, a member of the order Coleoptera (beetles) (Hinton and Hammock, 2003b). Members of this order are evolutionarily far removed from those in Lepidoptera although there is evidence that coleopteran JHE plays similar roles in larval development (Hirashima et al., 1995; Vermunt et al., 1997). Recombinant JHEs have also been expressed and biochemically characterized from a dipteran, *Drosophila melanogaster* (Campbell et al., 2001) and four lepidopterans, *H. virescens* (Hammock et al., 1990; Bonning et al., 1992), *C. fumiferana* (Feng et al., 1999), *M. sexta* (Hinton and Hammock, 2001), and *B. mori* (Hirai et al., 2002) as described above. In the following study, we analyze the sequence homology of JHE proteins with known, full-length cDNA sequences. Subsequently, we utilize the recombinant JHE enzymes from three species (*M. sexta*, *H. virescens*, and *T. molitor*) for comparative analysis. First, we address the question of whether there is a positive correlation between substrate and inhibitor specificity and amino acid sequence conservation. Substrate selectivity and inhibitor specificity experiments in this study were designed to probe the characteristics of the binding pocket of the enzyme. Second, we examine the stability in solvents and detergent of these JHEs, and third, a nonrecombinant JHE from the hemolymph of *T. ni*.

5. Materials and methods

5.1. Enzyme preparation

The recombinant JHEs from *T. molitor*, *M. sexta*, and *H. virescens* were produced in High 5 cells by the recombinant baculoviruses AcTmAJHE (Hinton and Hammock, 2003b), AcMs7JHE (Hinton and Hammock, 2003a), and AcUW2(B).JHE (Bonning et al., 1992), respectively. The High 5 cells were cultured in suspension in serum free medium (ESF-921, Expression Systems LLC). The cells were inoculated at a multiplicity of infection of five plaque forming units per cell, and the recombinant JHEs were purified from the culture supernatant at 48–60 h post-infection (h p.i.) after the supernatant was cleared of cells and cell debris by centrifugation at $2000 \times g$ for 10 min at 5 °C. In order to purify the JHEs of *T. molitor* and *M. sexta*, Tris–HCl was added to the cleared supernatants to a final concentration of 50 mM, pH 9.1, followed by incubation at 4 °C for 30 min prior to centrifugation at $10,000 \times g$ for 20 min. The supernatant containing the JHE of *T. molitor* was then concentrated using a Pall Filtron ultra filtration device with a 50 kDa MW cutoff (Pall Filtron Corp). This JHE preparation from *T. molitor* was used for substrate selectivity assays. For the inhibition and solvent stability studies, the JHE of *T. molitor* was prepared as described previously (Hinton and Hammock, 2003b). The supernatant containing the JHE of *M. sexta* was first diluted 1:4 with double-distilled H_2O (ddH_2O) followed by anion exchange chromatography on a 5 ml Q-Sepharose column (Amersham Biosciences). Greater than 98% of the detectable JHE activity bound to this column in one passage. The column was washed with 20 ml of 10 mM Tris buffer, and then the loaded protein was eluted stepwise in increasing NaCl. The majority of JHE activity eluted in the 200 and 350 mM NaCl fractions, which were further concentrated and desalted using a Centricon-30 filtration device (Millipore). In order to purify the JHE of *H. virescens*, the cleared supernatant was diluted 1:4 with cold 10 mM Tris buffer, pH 8.0, followed by anion exchange chromatography on a 5 ml Q-Sepharose column. Nearly 100% of the detectable JHE activity bound to the column in one passage, and the majority of this activity was eluted in the 200 mM NaCl fraction. This fraction was desalted and further concentrated in 50 mM phosphate buffer, pH 7.4, using a Centricon-30 filtration device (Millipore).

Nonrecombinant JHE from *T. ni* was collected from the hemolymph of larvae which had reached the second day of the fifth instar. The hemolymph was collected into a capillary tube and subsequently diluted in 100 mM phosphate buffer, pH 7.4, containing 0.01% phenylthiourea and cleared of hemocytes and other large macromolecules by centrifugation at $1000 \times g$ for 5 min.

5.2. Enzyme assays

All of the enzyme assays were performed with appropriate dilutions of the enzyme so that the rate of the observed product formation was linear within the time frame of the assay. JH hydrolysis was assayed by the method of Hammock and Sparks (1977) using tritiated JH III (17 Ci/mmol, New England Research Products) diluted in cold JH III (Sigma) to generate a final concentration of 5×10^{-6} M in ethanol. The enzyme assays were performed at 30 °C for 15 min. in triplicate, and each assay was repeated three times.

The hydrolysis of p-nitrophenyl acetate, α-naphthyl acetate, and other naphthyl derivatives by the JHEs was measured by spectrophotometric assays. For assays utilizing p-nitrophenyl acetate, 20 µl of enzyme solution was added to 278 µl of 50 mM phosphate buffer, pH 7.4, and the reaction was initiated by the addition of 2 µl of substrate (dissolved in ethanol) to give a final concentration of 5×10^{-4} M. The formation of p-nitrophenol was monitored at 405 nm using a Molecular Devices microtiter plate reader. For the conversion of mOD/min to µmol/min, a standard curve was generated using p-nitrophenol at a final concentration of 13.3–333 µM into the same buffer solution as was used in the assay. The assays utilizing α-naphthyl acetate and other naphthyl derivatives were performed in analogous manner with the addition of 0.065% Fast Blue RR dye to the reaction mixture. The formation of the diazonium product of α-naphthol was monitored at 450 nm and a standard curve was generated using α-naphthol at a final concentration 13.3–267 µM.

5.3. Inhibitor studies

All of the inhibition experiments were carried out using ³H-JH III as the substrate. These assays were performed as described above with the addition of 1 µl of a stock solution of inhibitor (dissolved in ethanol or DMF) to the reaction mixture. As a control reaction, 1 µl of solvent was added to the reaction mixture. The median inhibitor concentration (IC_{50}) was determined by regression analysis of at least four points (at least two points on each side of the IC_{50}) in the linear region of the curve. The assays were run in triplicate at each inhibitor concentration and the mean value of three separate curves was used to determine the IC_{50}. The TFK inhibitors were synthesized in this laboratory as previously described (Hammock et al., 1984). Abbreviations are as follows: PETFP (1,1,1-trifluoro-3-phenethylsulfanyl-propan-2-one), HTFP (1,1,1-trifluoro-3-hexylsulfanyl-propan-2-one), OTFP (1,1,1-trifluoro-3-octylsulfanyl-propan-2-one), DETFP (1,1,1-trifluoro-3-decylsulfanyl-propan-2-one), and DDTFP (1,1,1-trifluoro-3-dodecyl-sulfanyl-propan-2-one). The sulfone analogs are abbreviated with the "dOHSO$_2$" suffix indicating the hydrated state of the carbonyl.

5.4. Solvent and detergent effects

These experiments were done using the ³H-JH III partition assay as described above except the enzyme buffer consisted of 50 mM Tris–HCl, pH 7.8, and contained the appropriate amount of acetone, Triton X-100, ethanol or isopropanol. For each concentration, a control tube was added to ensure that JH III did not partition into the aqueous phase independently of enzyme hydrolysis under these conditions.

6. Results and discussion

In order to obtain a sense of hierarchy in the relative homologies among different esterases and to identify any sequence conservations that might be responsible for specific biochemical properties, protein homology alignments were made for the available JHE sequences in the GenBank databases. Fig. 3 shows the homology alignments of JHEs from three orders of insects including four lepidopterans, a coleopteran, and a dipteran as well as alpha E7 esterase from *Haematobia irritans* (Guerrero, 2000). The JHE sequences from the lepidopteran insects, including *M. sexta* (Hinton and Hammock, 2001), *H. virescens* (Hanzlik et al., 1989), *B. mori* (Hirai et al., 2002), and *C. fumiferana* (Feng et al., 1999) showed from 52% to 59% identity to each other. The lepidopteran JHEs showed only about 30% identity to the JHEs of *T. molitor* (Hinton and Hammock, 2003b) and *D. melanogaster* (Campbell et al., 2001), which showed 40% identity to each other. Amino acid residues consistent with a putative catalytic tetrad that is found in the catalytic site of the JHE of *H. virescens* (Ward et al., 1992; Thomas et al., 1999) were completely conserved in each of the esterases including alpha E7. Alpha E7 esterase showed the highest homology to the JHEs from *M. sexta* and *H. virescens*. By comparison of JHEs from the different insect orders, it was apparent that the JHEs as a group are not as highly conserved as was earlier thought. While these sequences do not establish clearly whether or not JHEs evolved early in the evolution of the class Insecta, they raise questions of how far the sequences can diverge while maintaining specific biochemical function. The most obvious sequence motif that was unique to the JHEs was GQSAG, in the immediate vicinity of the catalytic serine that has been found in all JHEs thus far cloned. There were relatively few sequence motifs that were common among all of the JHEs aside from this GQSAG motif. Some conserved structural properties, however, would be expected if JHE enzymes were to have specificity towards their assumed substrate (JH). If the binding pocket of the enzyme, into which a long hydrocarbon chain must fit, needs to only consist of hydrophobic amino acid residues, then many different amino acid residues could line the pocket of

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         1        10        20        30        40        50        60
MsJHE   MDARRQVCYYVLALLCVSELS...ARIPSTEEVVVRTESGWIRGLKRRAEGHKSYASFRGVPYAKQPLGE
BmJHE   MCTKFAVLLYYVIVGSVRAYSPAASPPSSCNVVAQTESGWVCGRTRRAEASTLYASFRGVPYAKQPVGE
HvJHE   MTSHVLALAFFLHACTALAWQ.....ETNSRSVLAHLDSGIIRGVPRSADG.IKFASFLGVPYAKQPVGE
CfJHE   MVSSVKSVAVALLVAVCGAAS.........ERLRGARCARRRAGCAARARAGGDYASFLAVPYAKQPLAA
TmJHE   .....MKLNVFVLISLHICFTLESVSTYAPKSPIVYTKYGPAIGLTEVSRSKREFMSFRGIPYAKPPVGE
DmJHE   .MLQLLLLGQLLAGPGPFCAALATVDQLTVCPPSVGCLKGTNLQGYQSER....FEAFMGLPYALPPIGD
HiAE7   MNFNVSFLEKLRWKIKCVENKILNYRLITNETHIVDTEYGKIKGVKRLTVYDDSFYSFEGIPYAKQPVGE

         70        80        90       100       110       120       130
MsJHE   LRFKELQPLEPWQDELDATQEGPVCQQTDVLYGRIMRPRGMSEACIHANIHVPYYALPRDAADKNRFA..
BmJHE   LRFKELQPAEPWTDYLDATEEGPVCYQTDVLYGSLMKPHGMDEACIYANIHVPLNALP.AAGETPTKP..
HvJHE   LRFKELEPLEPWDNILNATNEGPICFQTDVLYGRLMAASEMSEACIYANIHVPWQSLPRVRGTTPLR...
CfJHE   LRFQELQPAEPWKGCRDATKPGPICPQRDLFYGPTMRASEMSEACIHANVYHPLQYLPAASEGAAPRNRT
TmJHE   LRFAAPEPPEPWNFAINATQNGPICIQKNYFFSDPKVEG..TEDCLYLNVYVP.........KVKGTA
DmJHE   LRFSNPKVMPKLLGMYDASAPKMDCIQKNYLLPTPVVYG..DEDCLYLNVYRP...........EIR.KS
HiAE7   LRFKAPQRPVPWDGVKDCCHAASRSVQTDFISGN....SSGSEDCLYLNVYTN...........NLNTDT

        140       150       160       170       180       190       200
MsJHE   GLPVLVFIHGGGFAFGSGDSDLHCPEYLVSK.DVIVITFNYRLNVYGFLSLNST..SVPGNAGLRDMVTL
BmJHE   GLPIILVFIHGGGFAFGSGDADLYCPEYLVTR.NVVVITFNYRLNFFGFFSLDTP..KVPGNIGLFDMVTL
HvJHE   ..PIILVFIHGGGFAFGSGHEDLHCPEYLVTK.NVIVITFNYRLNVFGFLSMNTT..KIPGNAGLFDQVTL
CfJHE   LLPVLVYIHGGGFGFGSGDPDLHCPEYLSLMGD.NVVVITFNYRLNFFGFPGFLGFLSLMSS..SVPGNAGLFDMVTL
TmJHE   LLPVMLFIMGGGFLAGRGTSDYFCPEYIMDK.DVILVTFNYRLGVFGFFTTLDD..FAPGNYGLNDQVMA
DmJHE   ALPVNWYYIHGGGFGGCSAGPGVTCPENFMDSGEWILVTMAYRLGPFCFLSTQDA..VMSGNFGLKDQMLA
HiAE7   KRPVLVFFHGGGFICGEANRNYYCADYFIKK.DVVFITVQYRLGVLGLSLNSENLNVPGNAGLRDQVMA

        210       220       230       240       250       260
MsJHE   LKWVQRIAHFGGRPDDVTLMGQSAGAAATHILSLSKAADGLFRRAILMSGTSSSAFFTTNPVFAQYINK
BmJHE   LRWVKRIARAFGGDPDNVLAGQSAGAAAAHLLTLSKATEGLVVKRAILMSGAGTSTFFTTSPIFSQSINK
HvJHE   LRWVQRIAKNFGGDPSDITIAGQSAGASAAHLLTLSKATEGLFKRAILMSGTGMSYFFTTSPLFAAYISK
CfJHE   LRWVCRIARAFGGDPDNVLAGQSAGAASAHILSLSEAARGLFKRAILMSGTSTRSFFSTSPAYAQYVAQ
TmJHE   IKFVQETIECFGGDKNRVTIFGQSAGGGSVSLHLVSPLSRGLFQQAISQSGVALDLWAKPLNPLQVQITA
DmJHE   LRWVQRIIRFFGGDPQRVTIFGQSAGGVAAHMHLLSPRSHGLFHRVISMSGTANVPFAIAEQPLEQARLL
HiAE7   LRWIKINICASFGGDPDCILFGESAGAASTHYMMITEQARGLFHRAVLMSGTAMCIWAHTQCQHRGYTIA

        280       290       300       310       320       330
MsJHE   LFVTNIGITATDP..EEIHQKLIEMPAEKLNEANR..FLLEQFGLTTFFPVVVESP.INGVTTILDEDPEQ
BmJHE   ILFSILGVNSTNP..DEIHEKLVAMPVEKLNEANR..ILIDQIGLTTFFPVVVETP.HPGITTILDEDPNI
HvJHE   QLLQILGINETDP..EEIHRQLIDLPAEKLNEANA..VLIEQIGLTTFVPIVVESP.LPEVTTIILDDDPEI
CfJHE   LFLTNILGINGTDP..EEVHRLLIDTPIEKIIDAQN..DLQDVIGLTVFVPVVVESA.HPGVEIILDNDPEI
TmJHE   ALATFAGCQAQLANRADLLKCLREVDAQKLAETADKFKYFSIEPLTPYTLVTENKTAANPNPFLEKQPLS
DmJHE   AEFADVPDARNLS.TVKLTKALRRINATKLLNAGDGLKYWDVDHMTNFRPVVEEG..LEVDAFLNAHPMD
HiAE7   KRIGYKGENNDKD...VYDFLMKANPYDLAREEHKVLTNEELRDKVMFAFGPITTEPYETPDCVLPKPURE

        340       350       360       370       380       390       400
MsJHE   LIAKGRGKHTPLIIGFTDAECEIFRRQFEQIDIVSKIKENPGILVPLSVLFS...S.APDTVAEITKAMHE
BmJHE   LVQQGRGKDTPLIIGFTNSECHMFQBRFEQIDIVSKIUENPAILVPSNLLYS..S.TPETIALVSNQISQ
HvJHE   LIAEGRGKINTPLLIGFTSSECETFRNRLLNFDLVKKIQDNPTIIIPPKLLFM...T.PPELLMELAKTIER
CfJHE   LQSQGRGKDIPFLMCFTNAECQTFRPRFESIDIISRIAEQPVLVMPPPAIYV..T.PLDELPNKIGQIVQ
TmJHE   YLQDEEFLKVVPWIVGNVQDEGILRVAQLVRQPETLKALNDNFKTLITQLLALQVSVGEN.TTSLYDNMIN
DmJHE   HLAQGMPTSIPLLLGTVPGEGAVRVVNILGNETLRQSFNLRFDELLQELLEFPASFSQDRREKHMDLLVE
HiAE7   MLKTAWGNSIPTLIGNTSYEGLLFISVGKQNPHLIKELETFECYVPGELVVEDRS.S.PESLEIASILKK

        410       420       430       440       450       460
MsJHE   RYFKK........SVDMEGYIELCTDSYFMYPAISLAIKRA.RSNGAEVYLYQFSFDGDYSVFREVNHLN
BmJHE   RYFNG........SVDLEGFINMCTDSYYKYPAMKLAEKRS.AAGDAEVFLYQFSYDG.YDVFKQAFHLH
HvJHE   RYYNG........TISIDNFVKSCSDGFYEYPALKLAQKRA.ETGGAELYLMYPAYEGGQNSIIKKVMGLN
CfJHE   RYFNG........TPNLNKFIKLCSESYFVYPTLKLAEMRA.ANGGAEVYLMRFTYEADYSVFQEGLRLK
TmJHE   FYLGGKSSIDVNDPKSVQGFIDLYTDRSFTYGTYQTAILQS.DAGHTPIMMINEDYRGQYSYGDQFAATT
DmJHE   VYFQGQHEVN...ELTVQGFMNLISDRGFKQPLYNTIHKNV.CHTPNPVYLMSFNYQGPLSYASAYTSAN
HiAE7   LYVRG........ETPTLESFTELCSDFYFWYPMHRFLQLRFNHTVGSPIYLMRFDFDSEELINPYRIMRY

        470       480       490       500       510       520
MsJHE   .....FEGAGHIEDLTYVFRTNSMLGGHASFPPHDKDDHMKYWMTSFITNBMKYSNFVTDAK.LWPEVRA
BmJHE   .....FNGAGHADDLTYVLKVNSASG...TSSSQKADDEMKYWMTTFVTNGMRCSAEMCDETTAMKPVTP
HvJHE   .....HEGAGHIEDLTYVFKVNSMSEVLHASPSEN.DVKMKNLMTGYFLNGIKCSQPTCEDNNSLEVWPA
CfJHE   .....YRCTGHSEDLTLLFRANHVLG...ERAISPRDHKMVDAMTTYVTNGHLHSNPT.KGVAGWPAVKR
TmJHE   DDINFNWGVSHCDDLLYLFKSPALFP...DLEKE.SDLKMSEALVTHWTNGRIHGTEDPFNSLQWNSLNF
DmJHE   ..VTGKYGVVHCDDLLYLFRSPLLFP...DFQRNISTEAKVIHSFVDYFVHGAKFGHPR..NSVLLTPCSI
HiAE7   G..RGVKGASHTDDLTYLEWTMLSKR....MPKDSREYKTIERMIGIWTGQPATTGNEYSPEINGMENTTW

        530       540       550       560       570
MsJHE   DN.LRYQDIDTPDVYQNVKPHSEQRDMLDFFDSIYNWNGTSYCIK..........
BmJHE   RE.LQYQDIITPNLCHQTSLTKEQLEMKNFFDKIH..NGGESRLK..........
HvJHE   NNGMQYEDIVSPTIIRSKEFASRQQDIIEFFDSLS....SRSPLE.........
CfJHE   EK.LQYQNIITPELVT.TEVSRDLRDTMFFFDNIY....SSSS............
TmJHE   NNTEEIKGSNLKFLNMTGSSKTKT.IEFKLQRGFYPERMQFWANQNLAENFPGLG
DmJHE   EVLQSRPDGICDYHEFANAPDAYQGFEVHVASEFQTDRVNLWSHILNEK......
HiAE7   DS.LKKSDEVYKCMNIGDELKFIDLPEMEKLKVWQSVFNKKRELF.........
```
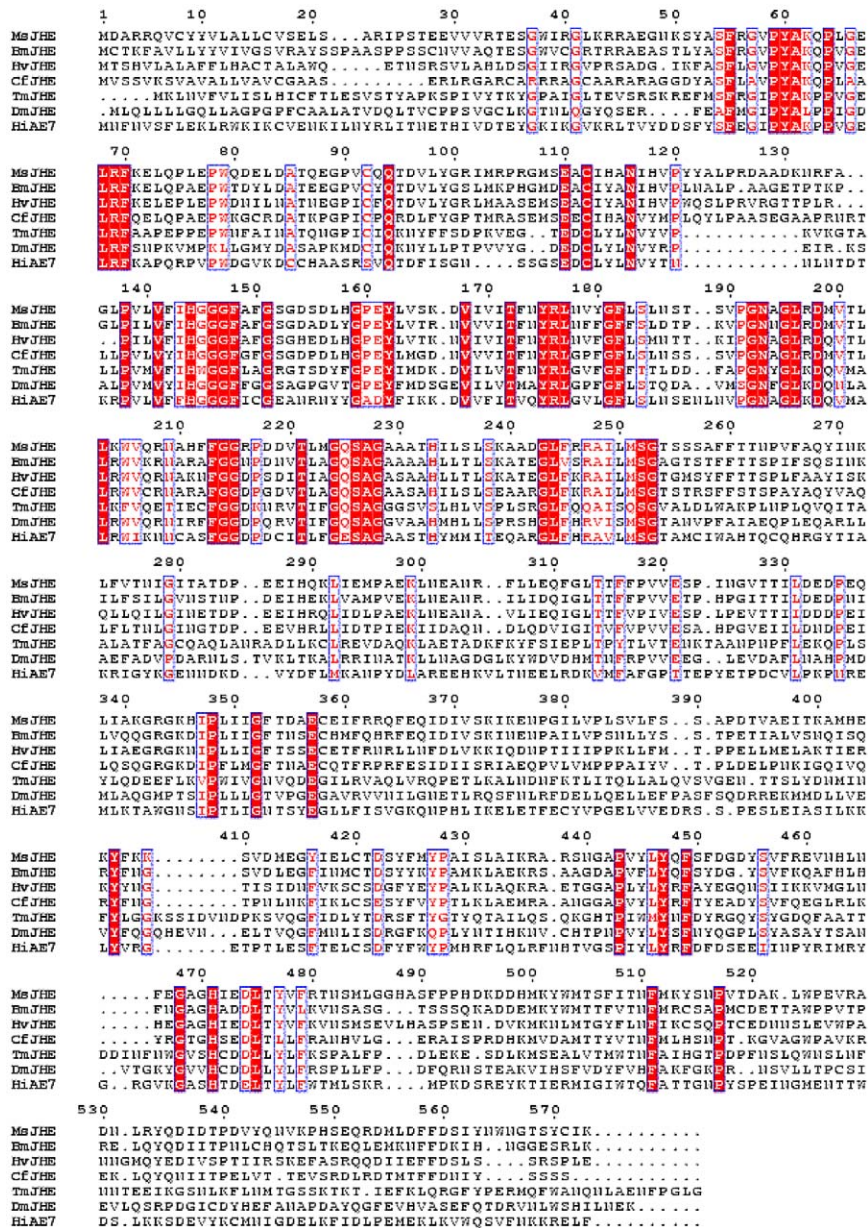
Fig. 3. Alignment of the deduced protein sequences of JHEs from *M. sexta*, *B. mori*, *H. virescens*, *C. fumiferana*, *D. melanogaster*, and *T. molitor*; and alpha E7 esterase from *H. irritans*. Amino acid residues that are conserved in all seven proteins or in all but one of the proteins are indicated by shaded and open boxes, respectively. The conserved GQSAG motif of the JHEs is underlined. Also, underlined are the putative members of the conserved catalytic tetrad, including the catalytic serine residue found within the GQSAG motif (Ward et al., 1992; Thomas et al., 1999).

the enzyme and retain similar hydrophobicity. Therefore, an overall hydrophobicity could be maintained while the specific amino acid sequence need not be so tightly conserved.

Comparative biochemical analyses of the three recombinant enzymes were done in order to uncover significant differences and/or similarities between them. Since two of the three JHEs used here are from more closely related species (*H. virescens* and *M. sexta* are in the order Lepidoptera while the third species, *T. molitor*, is in the order Coleoptera), any differences detected might be attributable to the evolutionary divergence of the two

species and/or different orders of insects. Table 1 shows inhibition of the three recombinant JHE enzymes by a series of TFKs with different chain lengths. This series of inhibitors was designed to mimic the transitional state of JH during enzymatic hydrolysis, with the extended hydrocarbon chain mimicking the terpenoid backbone of JH. OTFP, with a sulfur in the beta position (see Fig. 1) corresponding to the 2,3-olefin of JH and a carbon chain length of 8 carbons, closely resembles the JH backbone in length. In this study, all three enzymes were similar in that the most potent inhibitor of the series was DETFP (10 carbon chain), although IC_{50} values OTFP

Table 1
IC_{50} values of TFK inhibitors upon recombinant JHEs from *M. sexta*, *H. virescens* or *T. molitor*

Inhibitor[a]	Length of chain[b]	IC_{50} (nM)[c]		
		M. sexta	*H. virescens*	*T. molitor*
PETFP	Phenethyl	400 ± 12	75 ± 5.3	36 ± 1.6
HTFP	Hexyl	1700 ± 83	1100 ± 73	14 ± 1.1
OTFP	Octyl	9.1 ± 0.26	5.8 ± 0.26	4.6 ± 0.32
DETFP	Decyl	2.8 ± 0.22	0.99 ± 0.22	3.6 ± 0.26
DDTFP	Dodecyl	4.4 ± 0.34	2.2 ± 0.10	12 ± 0.11

[a] Abbreviations of the inhibitors, enzyme preparation, and assay conditions are described in the Materials and methods.

[b] Refers to the R moiety as shown in Fig. 1.

[c] IC_{50} is defined as the inhibitor concentration at which hydrolysis of JH III is 50% inhibited. The results shown are the means ± standard deviation of three separate experiments.

(8 carbon chain) and DDTFP (12 carbon chain) were each within an order of magnitude of that of DETFP for each enzyme. When the chain length was reduced to 6 carbons (HTFP), JHE of *T. molitor* remained sensitive, with IC_{50} in the 10^{-8} M range, while the sensitivity of each of the lepidopteran JHEs dropped by nearly three orders of magnitude. When the chain was altered to a phenethyl moiety (PETFP), the JHE of *T. molitor* was moderately sensitive, while those of *M. sexta* and *H. virescens* were much less sensitive. Overall, our results showed that DETFP was the most potent inhibitor for the three recombinant JHEs tested.

The majority of current IC_{50} assays are performed with TFK inhibitor dilutions prepared in ethanol. However, Wheelock et al. (2001) reported IC_{50} values for a new series of sulfone-containing TFKs that were assayed in DMF solutions. When these sulfone-containing TFKs were assayed in solutions prepared from ethanol, differences were found in the IC_{50} values (Table 2). The data from the DMF preparations showed large standard deviations (with RSDs as high as 61%), whereas the ethanol data consistently had lower deviations (RSDs ranged from 0.5% to 7%). Both studies used a solvent control

to account for direct effects upon the enzyme and reported that there were no significant effects upon enzyme activity. Thus, solvent effects upon either the inhibitor or the enzyme or some combination of both may account for these differences. The maximum amount of solvent present in any of the assays was 1%. This low level of solvent is unlikely to directly affect the structure of the inhibitor; however, the hydration state of the TFK may be affected. As discussed above, Wheelock et al. (2002) reported that inhibitor hydration state can substantially affect both inhibitor potency and binding kinetics. It is also possible that the presence of organic solvent affects the 3D structure of the enzyme and subsequent interactions between the enzyme and the inhibitor and/or substrate. Further studies are required to determine the exact nature of these solvent effects. These data show that the choice of solvent in preparing inhibitor dilutions can have a very large effect upon IC_{50} value. Therefore, comparisons between different bodies of work should be careful to be certain that inhibitor dilutions were prepared consistently.

Only a few reports have clearly shown that JHE is active against general esterase substrates such as naph-

Table 2
Effect of solvent upon the IC_{50} values of various inhibitors of JHE from the hemolymph of *T. ni*

Inhibitor[a]	Length of chain[b]	IC_{50} (nM) in ethanol[c]	IC_{50} (nM) in DMF[d]
PETFPdOHSO$_2$	Phenethyl	489 ± 22.3	1780 ± 570
HTFPdOHSO$_2$	Hexyl	147 ± 8.4	354 ± 20
OTFPdOHSO$_2$	Octyl	126 ± 0.6	147 ± 89
DETFPdOHSO$_2$	Decyl	43.6 ± 2.9	119 ± 23
DDTFPdOHSO$_2$	Dodecyl	62.1 ± 4.0	191 ± 9

[a] Abbreviations of the inhibitors, enzyme preparation, and assay conditions are described in the Materials and methods. All of the structures contain a sulfone (SO_2) beta to the carbonyl as shown in Fig. 1.

[b] Refers to the R moiety as shown in Fig. 1.

[c] IC_{50} is defined as the inhibitor concentration at which hydrolysis of JH III is 50% inhibited. The results shown are the means ± standard deviation of three separate experiments.

[d] Data taken from Wheelock et al. (2001).

thyl acetate, *p*-nitrophenyl acetate, and similar derivatives. Since JHE is generally found as a low abundance enzyme in vivo, preparations of JHE from biological sources have often been insufficient in terms of concentration of the JHE-specific enzyme. Early attempts to study JHE with the use of surrogate substrates were also misleading because of the high general esterase activity present in insect blood and tissues (Whitmore and Gilbert, 1972). Additionally, poor sensitivity of the assays involving these substrates seem to have limited most observations of activity with enzyme preparations of low specific activity. A few examples of highly purified JHEs have been reported with activity against *p*-nitrophenyl acetate and/or naphthyl acetate (Hanzlik and Hammock, 1987; Campbell et al., 1998; Zera et al., 2002). With our recombinant enzymes, strong viral promoters were used to drive JHE expression, and partial purification was relatively simple from the cell culture system. Thus, it was possible to prepare active JHE that was higher in terms of enzyme activity per milliliter than was available in many of the previous studies. By using a series of substrates, which differed in chain lengths, we attempted to observe substrate selectivity among the three JHEs with regard to the size of the acid component of the ester. The series of naphthyl analogs increased in size from acetate (C = 2) to propionate (C = 3) to butyrate (C = 4) to caproate (C = 6) to caprylate (C = 8).

As shown in Table 3, the three JHEs were similar in that their activity towards the naphthyl substrates decreased as the acid chain length grew from propionate to caprylate. With JHE of *T. molitor*, the activity dropped to barely detectable levels at naphthyl caproate, and the difference between hydrolysis against naphthyl caproate and naphthyl caprylate was insignificant. JHE of *M. sexta* differed from the other two JHEs in that the highest activity was against naphthyl acetate, and then activity decreased almost by half when tested with naphthyl propionate. By contrast, JHE of both *H. virescens* and *T. molitor* showed higher activity against naphthyl propionate than any other naphthyl derivative including

naphthyl acetate. The limited data from the *p*-nitrophenyl series also supported the trend that was shown with the naphthyl series, in that for each enzyme, the activity against *p*-nitrophenyl acetate was higher than the longer chain derivative, *p*-nitrophenyl valerate. This effect of chain length on rate of hydrolysis is widely observed among esterases. Deacylation, or k_3, of the acyl enzyme normally is the rate-increasing step. This rate generally decreases with increasing hydrophobicity or chain length. The limited data with the *p*-nitrophenyl derivatives also suggest a preference by JHE from *T. molitor* for *p*-nitrophenyl acetate over naphthyl acetate, which is not as clear with the lepidopteran JHEs. The ratio of activity for *p*-nitrophenyl acetate over naphthyl acetate was 1.9, 1.5 and 0.45 for JHEs from *T. molitor*, *H. virescens* and *M. sexta*, respectively.

Increased knowledge of the specificity of these enzymes may contribute to the development of new, improved surrogate substrates, and to determine guidelines for the application of such substrates to a complex mixture of enzymes. The hypothetical natural substrate, JH, has a small alcohol on one side of the ester bond, and a long chain lipophilic acid on the other side of it. Alternatively, with naphthyl acetate and *p*-nitrophenyl acetate, the acid component (acetate) is small and the alcohol is relatively large. If the JH binding pocket is designed to fit only a small alcohol and large acid, then these model substrates might not bind as well to the enzyme. However, once the substrate is bound, then hydrolysis should be very rapid.

The data obtained using the naphthyl derivatives and *p*-nitrophenyl derivatives must be analyzed with caution in regard to drawing a conclusion about the JH binding site, since the directionality of the acetate group with respect to the carbonyl may be variable. One assumes that the small acetate lies in the position of the methyl ester of JH and the large nitro-phenyl group is oriented similar to the terpene chain. However, compounds in this series with larger hydrocarbon chains might cause the molecule to become oriented in the same direction as JH.

Table 3
Hydrolysis of esterase substrates by recombinant JHEs from *M. sexta*, *H. virescens* or *T. molitor*

Substrate	Chain length of substrate	Specific activity (nmol/min/ml)		
		M. sexta	*H. virescens*	*T. molitor*
α-Naphthyl acetate	2	1087 ± 54	150 ± 11	279 ± 18
α-Naphthyl propionate	3	449 ± 24	324 ± 15	496 ± 39
α-Naphthyl butyrate	4	262 ± 8	174 ± 11	191 ± 7
α-Naphthyl caproate	6	187 ± 12	102 ± 6	36 ± 10
α-Naphthyl caprylate	8	150 ± 16	37 ± 12	43 ± 14
p-Nitrophenyl acetate	2	489 ± 23	230 ± 16	534 ± 36
p-Nitrophenyl valerate	5	104 ± 8	47 ± 6	122 ± 14

Each enzyme was purified as described in the text. Assay conditions for each substrate are described in Materials and methods. The results shown are the means ± standard deviation of three separate experiments.

There are two theoretical possibilities as to how naphthyl acetate could be bound to the enzyme. Firstly, the alcohol portion of the ester, which in this case is a large naphthyl group, could be forced into the binding pocket of the enzyme that normally binds to a small methyl group. In this case, the orientation of the alcohol and acid group towards the ester bond would be the same as that of JH. Alternatively, if such the binding pocket is too small to fit a naphthyl group, then it is conceivable that the substrate could be oriented in the opposite direction so that the active site encounters an ester bond in reverse, such that the smaller acid fits into the alcohol binding pocket and the large naphthyl group lies in the acid binding pocket.

There is corroborative evidence that the second option described above is taking place with some or all of the substrates, i.e., the ester bond sits in reverse orientation such that the bulky naphthol group lies in the hydrophobic binding pocket of JHE that normally binds the long aliphatic acid side chain of JH. Thus, with naphthyl acetate or naphthyl propionate, the small acid chain could lie in the methyl alcohol binding pocket of JHE. Moreover, if the binding pocket were small in order to fit a methanol group, it would be likely that increasing the chain length of the naphthyl derivative beyond propionate would eventually force a large acid group into a small binding pocket and therefore activity would decline as both substituents of the ester are too large to fit well. The data shown in Table 3 support this hypothesis, because activity decreases for all three enzymes after the chain length is increased beyond that of propionate. Otherwise, if the acid component were bound into the JH acid binding pocket of the enzyme, one would expect the binding/activity to increase with increasing chain length, since a longer side chain would better mimic JH. This theory has been supported by inhibitor studies with TFKs for which longer chain length enhances inhibitor potency. Using the JHE from *H. virescens*, McCutchen et al. (1993) also showed a positive correlation between increasing chain length and hydrolytic activity in a series of thioether compounds in which the acid moiety is increasing in size with a small alcohol. The results from the current study, however, show that the longest chain derivative, naphthyl caprylate (C = 8) (in which the acid moiety is increasing in size with a large alcohol) had the lowest activity in the series. Enzymologists define specificity as k_{cat}/K_m. The high specificity of JHE for its substrate is driven by a very low K_m rather than a large k_{cat}. The conditions of these assays demonstrate that JHE can turn some surrogate substrates over well. However, these substrates are not selective for detecting JHE among other esterases, nor are they specific for JHE in kinetic terms.

Several studies have shown a preference by JHEs for the natural methyl ester and/or ethyl ester of JH over higher homologs of JH esters. Studies by Weirich and Wren (1973, 1976) with JHEs from *T. molitor*, *M. sexta* and *Samia cynthia* show that these enzymes hydrolyze the ethyl ester but not the propyl ester of JH I. Hammock et al. (1977) reported that JHE from *Blaberus giganteus* (Dictyoptera) is unable to hydrolyze ethyl ester analogs of JH. However, these authors may have inverted the 2,3-olefin of JH from the *trans* to the *cis* orientation during the synthesis of the ethyl ester used in their assays. A subsequent report by Grieneisen et al. (1997) demonstrates that JHEs from *M. sexta*, *H. virescens*, *T. molitor* and *Schistocerca americana* (Orthoptera) hydrolyze methyl and ethyl esters of JH I at similar rates; hydrolysis of the propyl ester and butyl ester are also detected, but at significantly lower rates. Retesting the JHE of *B. giganteus* with JH I analogs also resulted in hydrolysis of the ethyl ester at significantly lower rates than the natural methyl ester, while no hydrolysis of the propyl or butyl esters are detected (Grieneisen et al., 1997). The differences in binding between the methyl esters and ethyl esters of JH in the latter would likely have been more apparent if the assays were done at lower substrate concentrations. At high concentrations of substrate, most of the enzyme becomes bound and the k_{cat} becomes more significant as the rate limiting factor. In the case of JH, this involves a relatively large kinetic barrier with the deacylation step (involving the large hydrophobic portion of JH), for which there should be no detectable difference between the two JH esters. When considering the significantly decreased hydrolysis of the propyl esters in all cases, however, these results suggest that the limited size of the binding pocket for the alcohol group of JH results in steric hindrance. On the other hand, these studies as well as the current one are limited in that they only measured total hydrolytic rate instead of specifically obtaining K_m or k_{cat} values. When JH is used at high concentration, it is not clear whether the total rate is affected more by increasing K_m values or decreasing k_{cat} as the size of the alcohol moiety increases. Increasing the chain length beyond 8 carbons in this naphthyl series was difficult due to solubility problems. The substrates required emulsifiers or organic solvents in order to solubilize, and these conditions would not be suitable for all of the enzymes involved in the comparisons. It is also possible that as the chain length increases towards naphthyl caprylate, there is also increasing micelle formation during the course of the assay. Thus, the observed decrease in activity may have been partially dependent on substrate availability.

In a recent report by Zera et al. (2002), the JHE from the cricket *Gryllus assimilis* (order Orthoptera) was isolated and characterized. Interestingly, the JHE of *G. assimilis* shows higher selectivity towards longer chains of naphthyl derivatives (nonanoate > acetate), and a relatively low K_m for naphthyl acetate. These data led them to propose the possibility of multiple roles for the enzyme aside from JH hydrolysis. In order to test the

longer chain substrates, they used higher organic solvent concentrations than were used in this study and this may account for the observed differences. The JHE from *G. assimilis* may also be similar to the lepidopteran JHEs in terms of its stability in the presence of organic solvents and detergents as discussed below. Another unique feature of the orthopteran JHE is that it is found as a dimer, with relatively large mass for each subunit. The JHE of *G. assimilis* also shows a relatively high IC$_{50}$ value for OTFP.

Fig. 4A,B shows the effects of increasing concentrations of acetone and Triton X-100, respectively, on the hydrolysis activity of the three recombinant JHEs and nonrecombinant JHE from the hemolymph of *T. ni*. In previous reports, the JHE of *M. sexta* showed unique properties such as activation in organic solvents and stability in low concentrations of detergents that were thought to set it far apart from the other JHEs thus far characterized. This activation of hydrolysis activity was again found in this study. Although the JHEs from *H.*

virescens and *T. ni* did not show the same striking activation levels as those of *M. sexta*, these lepidopteran JHEs did show some enzyme activation at the lower organic solvent and detergent concentrations, and were in general, quite stable at higher solvent and detergent concentrations. This was in sharp contrast to the coleopteran JHE that showed a rapid decrease in enzymatic activity even at lower solvent and detergent concentrations. Similar trends were observed when increasing concentrations of ethanol (Fig. 4C) or isopropanol (Fig. 4D) were used as co-solvents. Although only one nonlepidopteran JHE was analyzed in this study, previous reports (e.g., Kramer and de Kort, 1976; McCaleb et al., 1980; Stauffer et al., 1997) have shown that JHEs from coleopterans such as *Leptinotarsa decemlineata*, *Ips typographus*, and *T. molitor* are completely or partially inhibited by low (e.g., 0.1%) concentrations of Triton X-100. Caution, however, must be taken with these observations because the assay used in these studies measured the formation of JH acid upon hydrolysis of the methyl
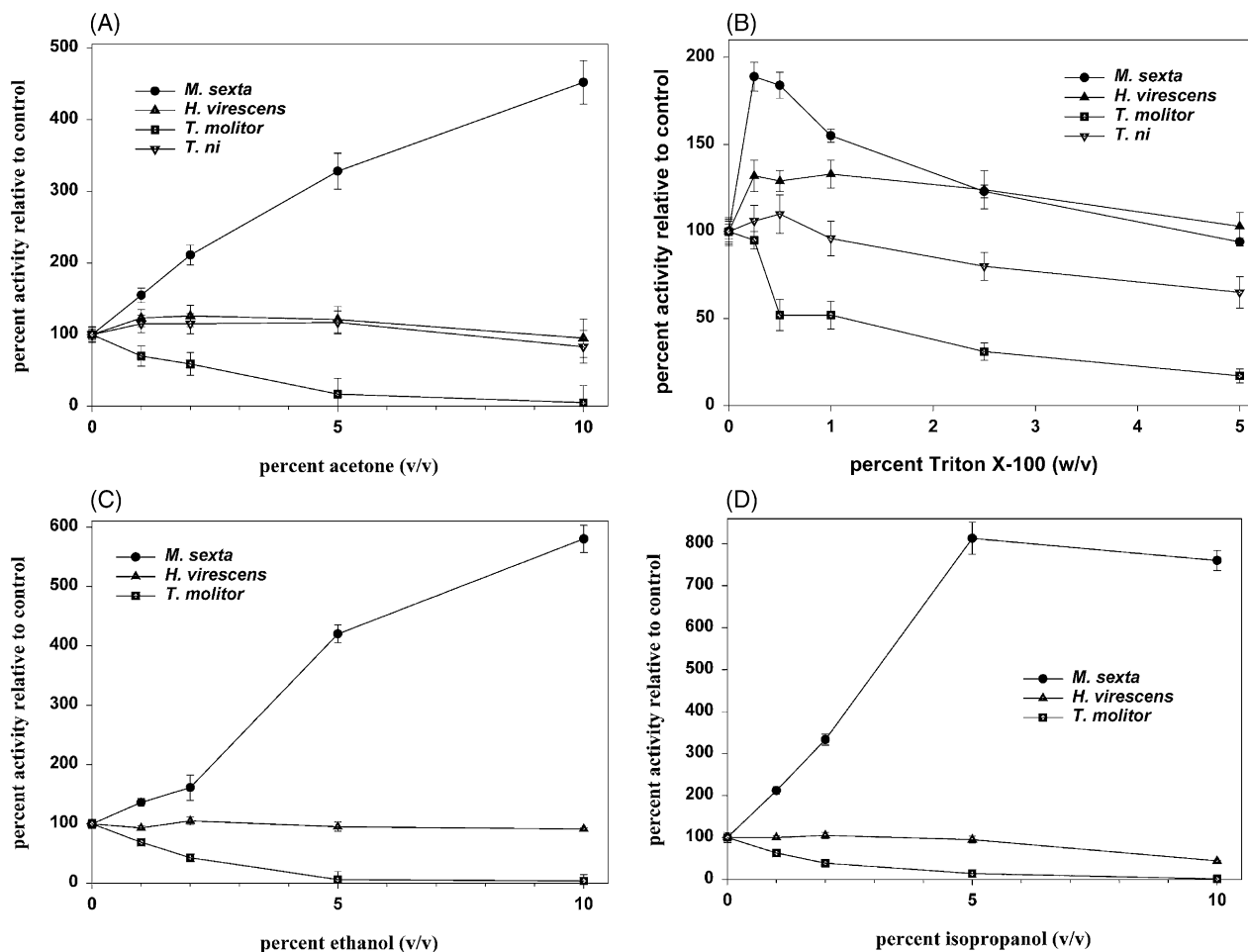

Fig. 4. Effects of co-solvents or detergent on JHE-catalyzed hydrolysis of JH III. The enzymes were diluted in Tris–HCl, pH 7.8, prior to addition of the co-solvent or detergent. Activity is expressed in comparison to control JHE in Tris–HCl buffer with no solvent or detergent added. Assays were performed in triplicate and the vertical bars indicate the standard deviation of the mean. (A) Effect of acetone as a co-solvent. (B) Effect of Triton X-100. (C) Effect of ethanol. (D) Effect of isopropanol.

ester by a partition of aqueous and organic layers. In the presence of a solvent such as acetone, enzymatic activity towards the ester bond of JH would be detected as JH hydrolysis. However, in the presence of higher alcohols, the enzyme activity may simply be shifted towards trans-esterification, which results in the replacement of a methyl ester with a less hydrophilic ethyl ester or propyl ester. Thus, differences in stability are mixed in with differences in substrate selectivity, and the JH partition assay may not be appropriate. Grieneisen et al. (1997) reported that the JHE enzymes are actually capable of transesterification in the presence of ethanol or 1-propanol.

In conclusion, this study showed that the lepidopteran and coleopteran JHEs are generally similar in terms of their selectivity towards substrates and inhibitors based on chain length. The coleopteran JHE was highly sensitive to low concentrations of organic solvents and detergents, while the lepidopteran JHEs were less sensitive or even activated. These findings may hint at possible trends in biochemical properties that are correlated to conservation of protein sequence. However, our observations are only based upon a small sample size of three recombinant JHEs. Recently, a JHE has been cloned from the dipteran species *D. melanogaster*. Because the sequence homology between JHEs of *D. melanogaster* and *T. molitor* are higher to each other than to the JHEs of the Lepidoptera, it will be interesting to see how the JHE of a dipteran biochemically compares with those of a coleopteran and lepidopterans and furthermore how any similarities in biochemical properties might be related to sequence homologies. In the future, significantly more detailed models of JHE structure will become available through crystallization studies and homology modeling as described above. With better structural models, more appropriate substrates and improved inhibitors can be designed for comparative biochemical studies such that a better understanding of the relationship between structure and function can be determined. Subsequently, site-directed mutagenesis techniques can be used to (i) verify the role of that specific amino acid residues play in functional activity and (ii) genetically engineer esterase enzymes with combinations of desired properties such as greater stability in vivo, greater stability under specified in vitro conditions, or enhanced substrate specificity. With these tools and information we will truly be able to answer the question of why JHE is so JH specific.

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