ABSTRACT

ANSPAUGH, DOUGLAS DAY. Molecular Genetics and Enzyme Regulation of Epoxide Hydrolases in the Cabbage Looper, *Trichoplusia ni*. (Under the direction of Dr. R. Michael Roe.)

A full-length epoxide hydrolase (EH) cDNA (TmEH-2) was isolated from a *Trichoplusia ni* cDNA library made from the digestive system of fifth (last) stadium larvae. When TmEH-2 was compared to a previously reported *T. ni* EH cDNA (TmEH-1) from fat body, the two were 67 and 73% identical at the nucleic acid and amino acid levels, respectively. In a phylogenetic tree of EHs inferred from amino acid sequence alignments, TmEH-1, TmEH-2 and other known insect EHs were more closely related to the microsomal than soluble EHs of other organisms. The tree not only separated microsomal from soluble EHs, but also grouped all insect EHs into one microsomal cluster. Contrary to earlier conceptions, it was determined in multiple sequence alignments that not all membrane-bound microsomal EHs (mEHs) contain identical amino acid residues at the sites of the proposed catalytic triad. While the *T. ni* EHs possessed the typical Asp-Glu-His triad of mEHs, the residues were Asp-Asp-His at the same positions in two of three previously reported *Drosophila melanogaster* mEH cDNAs. This soluble-like EH triad also was discovered in mEHs from two bacterial species. To determine the tissue specificity of TmEH-1 and TmEH-2 expression, total RNA from *T. ni* fat body or gut was assayed by Northern blot using TmEH-1 and TmEH-2 gene-specific probes. Although TmEH-1 and TmEH-2 were isolated from fat body and gut, respectively, each was expressed in both tissues. Also, both EHs were expressed in a similar, age-specific manner. TmEH-1 was subcloned into a baculovirus system for *in*

vivo expression in *T. ni* larvae. Following injections with non-occluded virus, the time to lethality of the TmEH-1 baculovirus was shorter than the non-transformed, control virus. At 72 h post-injection, the cumulative mortality for the TmEH-1 and control baculoviruses was 95 and 41%, respectively. The mechanism of accelerated toxicity of the TmEH-1 baculovirus currently is unknown.

Juvenile hormone (JH) III esterase and JH III EH *in vitro* activity was compared in whole body *T. ni* homogenates at each stage of development. While activity of both enzymes was detected at all ages tested, JH esterase was significantly higher than EH activity at all time points except day three of the fifth stadium. For both enzymes, activity was highest in eggs. Interestingly, adult virgin females had 4.6- and 4.0-fold higher JH esterase and EH activities, respectively, than adult virgin males. JH III metabolic activity also was measured in whole body homogenates of fifth stadium *T. ni* that were fed a nutritive diet (control) or starved on a non-nutritive diet of alphacel, agar and water. With larvae that were starved for 6, 28 and 52 h, EH activity per insect equivalent was 48, 5 and 1%, respectively, of the control insects. At the same time points, JH esterase levels in starved *T. ni* were 29, 4 and 3% of insects fed nutritive diet. Selected insect hormones and xenobiotics were administered topically or orally to fifth stadium larvae for up to 52 h, and the effects on whole body EH and JH esterase activity were then analyzed. JH III induced JH III esterase as high as 2.2-fold, but not JH III EH activity. The JH analog, methoprene, increased both JH esterase and EH activity as high as 2.5-fold. The JH esterase inhibitor, 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP), had no impact on EH activity. The epoxides, *trans*- and *cis*-stilbene oxide (TSO and CSO) each induced EH between 1.9- and 2.0-fold. TSO did not alter JH esterase levels when topically applied, but oral adminstration reduced activity to 70% of the control at

28 h, and then induced activity 1.8-fold at 52 h after treatments began. CSO did not have an impact on JH esterase activity. Phenobarbital increased EH activity by 1.9-fold, but did not change JH esterase levels. Clofibrate and cholesterol 5α,6α-epoxide had no effect on EH. JH esterase activity also was not affected by clofibrate, but cholesterol 5α,6α-epoxide was inhibitory to JH esterase, lowering activity to 60-80% of the control. The biological significance of these results is discussed.

MOLECULAR GENETICS AND ENZYME REGULATION OF EPOXIDE HYDROLASES IN THE CABBAGE LOOPER, *TRICHOPLUSIA NI*

by

DOUGLAS DAY ANSPAUGH

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APPROVED BY:

R. Michael Roe (Chair of Advisory Committee)	Ernest Hodgson
Randy L. Rose	Michael D. Tomalski

DEDICATION

This dissertation is dedicated to my parents,

Fred and Lou Anne Anspaugh.

Thank you Mother and Dad for always being there to listen, support, and encourage.

BIOGRAPHY

Douglas Day Anspaugh was born in Shelbyville, Indiana on April 29, 1966. He graduated from Shelbyville Senior High School in 1984, and then received a Bachelor of Science degree in Entomology from Purdue University, West Lafayette, Indiana, in 1989. During his undergraduate studies, Douglas completed two internships – one with Dow Chemical USA (Indianapolis, Indiana) and another with Mobay Chemical Corporation (Kansas City, Missouri). Upon graduation he was employed as a Research Associate Intern at Dow AgroSciences (formerly Eli Lilly & Co.; Indianapolis, Indiana). In 1990 he accepted a Research Technician position in the Department of Entomology at North Carolina State University, Raleigh, North Carolina. During this employment, he completed a Master of Science degree in Toxicology at NC State University in May 1995, and then pursued a Ph.D. in Toxicology. Both of his graduate degrees were under the direction of Dr. R. Michael Roe in the Department of Entomology at NC State. For his doctoral research, Douglas received the John Henry Comstock Graduate Student Award from the Southeastern Branch of the National Entomological Society of America in November, 2002. He also was selected as the 2002 Outstanding Ph.D. Student by the North Carolina Entomological Society. Following an August 2003 graduation, he will be employed as a Research Biologist in the Insecticide Discovery Group of the Agricultural Products Research Division at BASF Corporation, Research Triangle Park, North Carolina. Douglas will be married to Michelle Marie Kellogg in Shelbyville, Indiana on October 4, 2003.

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Personally, I am thankful for the support and encouragement throughout the years from my Mother and Dad, my brothers (Jeff, Rod and Chris) and their families, my Grandmother ("Nan"), and all of my relatives. I also appreciate the support and camaraderie of my friends during the course of this work, especially that of Chris Wilson. Furthermore, I am thankful for the friendship and heartening humor of Cullen Knott. Finally, I am very grateful for the companionship, understanding and patience of my fiancée, Michelle Kellogg. Thank you Michelle for listening, and inspiring me to keep going, especially when the going got tough.

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CHAPTER 1

MOLECULAR CHARACTERIZATION OF EPOXIDE HYDROLASES FROM THE CABBAGE LOOPER, *TRICHOPLUSIA NI*: PHYLOGENY, DEVELOPMENTAL EXPRESSION, AND CLONING FOR ENHANCED BACULOVIRUS TOXICITY

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ABSTRACT

A full-length epoxide hydrolase (EH) cDNA (TmEH-2) was isolated from a *Trichoplusia ni* digestive system cDNA library made with equal proportions of L5D1 and L5D2 larvae. TmEH-2 was 2054 base pairs (bp) in length with a 1389 bp open reading frame that encoded a putative polypeptide of 463 amino acids. In sequence alignments, TmEH-2 differed from a *T. ni* EH cDNA (TmEH-1) from fat body that was previously cloned in our laboratory (VanHook Harris et al., 1999. Insect Molecular Biology 8 (1), 85-86). The two EHs were 67 and 73% identical at the nucleic acid and amino acid levels, respectively. In a phylogenetic tree of EHs inferred from amino acid sequence alignments, TmEH-1 and TmEH-2 were more closely related to the microsomal than soluble EHs of other organisms. The tree not only separated microsomal from soluble EHs, but also grouped all insect EHs into one cluster within the microsomal EHs (mEHs). Contrary to earlier conceptions, we determined in multiple sequence alignments that not all membrane-bound mEHs contain identical amino acid residues at the sites of the proposed catalytic triad. While the *T. ni* EHs possessed the typical Asp-Glu-His triad of mEHs, the residues were Asp-Asp-His at the same positions in two of three previously reported *Drosophila melanogaster* mEH cDNAs. This soluble-like EH triad also was discovered in mEHs from two bacterial species. To determine the tissue specificity of TmEH-1 and TmEH-2 expression, total RNA from *T. ni* fat body or gut was assayed by Northern blot using TmEH-1 and TmEH-2 gene-specific probes. Although TmEH-1 and TmEH-2 were isolated from fat body and gut, respectively, each was expressed in both tissues. Also, both EHs were expressed in a similar, age-specific manner. Expression was highest on L5D0 and then diminished from L5D1 through L5D4. TmEH-1 was

subcloned into a baculovirus system for *in vivo* expression in *T. ni* larvae. Following injections with non-occluded virus, no signs were evident for enhanced juvenile hormone (JH) metabolism via epoxide hydration in insects treated with TmEH-1 baculovirus, when compared to treatments with non-transformed control baculovirus. Surprisingly, however, the time to lethality of the TmEH-1 baculovirus was shorter than the control virus. At 72 h post-injection, the cumulative mortality for the TmEH-1 and control baculoviruses was 95 and 41%, respectively. The mechanism of accelerated toxicity of the TmEH-1 baculovirus currently is unknown.

INTRODUCTION

Epoxide hydrolases (EHs) (EC 3.3.2.3) belong to a diverse group of enzymes known as the α,β-hydrolase fold family (Ollis et al., 1992; Lacourciere and Armstrong, 1994). EHs metabolize epoxides by hydration of the oxirane ring to produce *trans* diols. With mammals, a large number of reports are available on the role of EHs in the metabolism of xenobiotic (for e.g.: Oesch, 1973; Guenthner and Oesch, 1981; Guengerich, 1982; Seidegard and DePierre, 1983; Wixtrom and Hammock, 1985; Lake et al., 1987; Meijer and DePierre, 1987 and 1988) and endogenous epoxides (Halarnkar et al., 1989 and 1992; Nourooz-Zadeh et al., 1992). Much less is known about the function of EHs in insects. Previous work has shown that insect EHs can metabolize xenobiotic epoxides such as cyclodienes (Brooks et al., 1970; Brooks et al., 1974; Slade et al., 1975; Brooks et al., 1977). Additionally, EHs likely are involved in detoxifying dietary plant epoxides (reviewed by Mullin, 1988), and may have a role in metabolizing endogenous epoxides such as juvenile hormone (JH) (Hammock, 1985; Roe and Venkatesh, 1990).

The importance of JH metabolism in regulating insect growth and development (as well as many other physiological events) has been well documented (see reviews by: Roe and Venkatesh, 1990; Nijhout, 1994; Gilbert et al., 2000). Originally, it was conceived that the primary route of JH metabolism was JH esterase. EHs were assumed to be of secondary importance as an ultimate scavenger of JH acid, the product of JH esterase metabolism. Based on this hypothesis, past research focused on JH esterase. However, recent studies have suggested that EHs in some insects may be as important as JH esterase in the primary metabolism of JH (reviewed by de Kort and Granger, 1996). In fifth stadium *Trichoplusia ni*, for example, Kallapur et al. (1996) discovered in several

tissues that there were no significant differences between the peaks of JH esterase and EH activity against JH *in vitro*. Further evidence of a primary role for EH in JH metabolism was presented by Halarnkar et al. (1993). This study determined that a phosphate conjugate of JH diol, not JH acid-diol, was the principle end-product after *Manduca sexta* were injected with JH I.

While a number of EH genes from mammals and plants have been sequenced and expressed beforehand, much less is known about the molecular genetics of insect EHs. Only recently were the first insect EH cDNAs sequenced and expressed from eggs of *M. sexta* (NCBI access. #U46682; Wojtasek and Prestwich, 1996; Debernard et al., 1998), and from the fat body of *T. ni* (TmEH-1; NCBI access. #U73680; VanHook Harris et al., 1999). From each of these lepidopterans the expressed microsomal EH (mEH) appeared to be specific for JH metabolism. More recently, EHs have been cloned and sequenced from three other insect species, including the cat flea (*Ctenocephalides felis*; Keiser et al., 2002; NCBI access. #AF503908 and #AF503909), yellow fever mosquito (*Aedes aegypti*; unpublished; NCBI access. #AF517544) and the fruit fly (*Drosophila melanogaster*; unpublished; NCBI access. #AF517545, #AF517546 and #AF517547). Multiple EH cDNAs within an insect species were first isolated and sequenced from *C. felis* and *D. melanogaster*. There has been some evidence in the past that *T. ni* may have multiple EH genes, based on JH metabolism studies (Kallapur et al., 1996) and Southern blot analysis of genomic DNA with multiple restriction endonucleases (VanHook Harris, 1999). However, this conclusion had not been validated with nucleic acid sequence data from multiple EH cDNAs.

In the present study we report the cloning and sequencing of a second, unique EH cDNA (TmEH-2; NCBI access. #AF035482) from the digestive system of *T. ni*. The

relatedness of TmEH-2 to that of the first discovered *T. ni* EH (TmEH-1) and to EHs from other organisms was deduced in a phylogenetic tree inferred from amino acid sequence alignments. The tissue- and age-specificity of TmEH-1 and TmEH-2 RNA expression was determined with gene-specific probes in Northern blots. Lastly, to explore the role of EH in JH metabolism, TmEH-1 was subcloned into baculovirus for *in vivo* expression in *T. ni* larvae.

MATERIALS AND METHODS

Insects

Cabbage loopers, *Trichoplusia ni* (Lepidoptera: Noctuidae), for this study originated from the Shorey strain (California). Larvae were reared on an artificial diet (Roe *et al*., 1982) at 27 $\pm$ 1^{o}C with a relative humidity of 50 $\pm$ 5%, and a 14 h light:10 h dark cycle. The ages of gate 1, last (fifth) stadium larvae were determined as previously described (Kallapur *et al*., 1996). In our laboratory, the fifth stadium of this strain lasted 4 days, which were designated L5D1, L5D2, L5D3 and L5D4. The time immediately following ecdysis to the fifth stadium was called L5D0. Larvae wandered on L5D3, became prepupae on L5D4 and underwent ecdysis to pupae by the next day.

cDNA Library Construction

An equal proportion of L5D1 and L5D2 *T. ni* larvae were used to construct a cDNA library from the digestive system, which included fore-, mid- and hindgut (hereinafter referred to as gut). After guts were excised, any remaining malpighian tubules, tracheae and connective tissues were removed and the gut contents were purged with sodium phosphate buffer (0.1 M, pH 7.4). Total RNA from guts was then isolated according to the method of Chomczynski and Sacchi (1987). Poly (A^{+}) RNA was purified using a Poly(A) Quik mRNA Isolation kit (Stratagene, La Jolla, CA). The cDNA library was constructed using Zap Express cDNA Synthesis and Gigapack II Gold Cloning kits (Stratagene) according to the manufacturer's instructions.

cDNA Library Screening

The *T. ni* gut cDNA library was screened using the high-stringency procedure of Israel (1993). Basically, the phage library was aliquoted into an 8 x 8 matrix of a sterile microtiter plate at a concentration of 2000 plaque-forming units (pfu) per well. The phage were propagated and then pooled across columns and down the rows of the matrix to yield 16 phage pools. Aliquots from each pool (0.5 µl) were used as templates in 16 different DNA amplification reactions using the primer 5'AGTCTCTGTCCTTGCTG3' (EHRP2), and the universal M13R primer. EHRP2 corresponds to nucleotides 66-82 of $TmEH_{(237)}$ (NCBI access. #U35736), a 237 base pair (bp) putative EH cDNA fragment isolated by RT-PCR of total RNA from L5D3 *T. ni* (VanHook Harris et al., 1999). The conditions for DNA amplifications were 1 x reaction buffer (Promega, Madison, WI), 1 µg/µl bovine serum albumin, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 1.0 pM each primer and 1.0 unit Taq DNA polymerase (Promega) in 25 µl total volume. The thermal cycling conditions were 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 45°C for 3 min and 72°C for 2 min. The last cycle was followed by a final extension at 72°C for 7 min. Five µl of each amplification reaction were analyzed by agarose gel electrophoresis. The DNA was then denatured, neutralized and transferred to Hybond-N^+ nylon membranes (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. Nylon membranes were UV crosslinked in a Bio-Rad GS Genelinker (Bio-Rad, Hercules, CA) after transfer. The nylon membranes were pre-hybridized at 50°C for 30 min in Rapid-Hyb buffer (Amersham), and then hybridized at 50°C for 90 min with $TmEH_{(237)}$ that was random-prime labelled with ^{32}P-dCTP (Du Pont NEN, Boston, MA) using the Redi-Prime kit (Amersham). Also at 50°C, the membranes were washed twice in 2 x saline

sodium citrate (SSC), 0.2% sodium dodecyl salt (SDS) for 10 min per wash and then twice in 0.2 x SSC, 0.2% SDS for 15 min per wash. The blots were analyzed by autoradiography for the DNA amplification product of the expected size. By matching the positive signals obtained from pooled rows and columns of phage, several wells in the microtiter plate were identified as potentially containing the desired cDNA clone. The phage in one of these wells were then diluted and distributed into another 8 x 8 matrix at a concentration of 56 pfu/well. The entire DNA amplification and Southern blotting procedure was repeated. The microtiter plate wells of this second matrix were identified that potentially contained the desired cDNA clone. The phage in one of these wells were then diluted and distributed into a third 8 x 8 matrix at a concentration of 4 pfu/well, followed by another round of DNA amplification and Southern blotting. From the third matrix, phage from the appropriate well were plated on NZYM agar plates and screened by traditional library screening procedures (Sambrook *et al*., 1989) using ^{32}P-labelled $TmEH_{(237)}$ as a probe. This last step of conventional screening produced a single clone. The phagemid containing the desired EH cDNA was excised according to Stratagene's recommendations and transferred into the *E. coli* XLOLR strain. The resultant plasmid pGUT-EH was isolated with a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) and sequenced in both directions. The full-length EH insert (NCBI access. #AF035482) in pGUT-EH is referred to as TmEH-2.

DNA Sequencing and Sequence Analysis

DNA sequencing was performed by the DNA Sequencing Core Laboratory of the Interdisciplinary Center for Biotechnology Research, University of Florida (Gainesville, FL). The sequenced cDNA from *T. ni* gut was identified as an epoxide hydrolase by the

Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov; National Institutes of Health, Bethesda, MD). The amino acid sequence was obtained by computational translation of the EH cDNA using Wisconsin Genetics Computing Group (GCG, Madison, WI) SEQWeb Sequence Analysis software, version 1.1. GAP analyses by SEQWeb were utilized to calculate all reported percentage identities between EH DNA or protein sequences.

The phylogenetic relationships of EHs from a variety of organisms were derived by aligning amino acid sequences (obtained from NCBI Databases; www.ncbi.nlm.nih.gov/Database/index.html; National Center for Biotechnology Information, Bethesda, MD) with CLUSTAL X version 1.64b software. The phylogenetic tree was inferred from the resulting alignment based on uncorrected pairwise sequence differences using the neighbor-joining algorithm in the CLUSTAL X software package. As a measure of statistical validity of each node in the phylogenetic analysis, the bootstrapping method (Efron and Gong, 1983; Felsenstein 1985) was utilized.

Design of Gene-Specific EH Probes

A DNA probe was designed from each of the TmEH-1 and TmEH-2 cDNAs by restriction endonuclease (RE) digestion of the respective plasmids, pG6-1 and pGUT-EH. RE enzymes were chosen to liberate a fragment from the 3'-untranslated regions of each cDNA. Digestion reactions included 15.5 μg of plasmid, 20 units of each RE enzyme, and 1 x Multicore buffer (Promega). Reactions were conducted at 37^{o}C for 2 h. *Nco* I and *Hpa* I (Promega) were used to yield a 205 bp product (TmEH-$1_{(205)}$) from TmEH-1 (pG6-1). *Eco*R I and *Dra* I (Promega) digestion of TmEH-2 (pGUT-EH) produced a 327

bp fragment (TmEH-$2_{(327)}$). The desired fragments from digests were separated by agarose gel (2.0%) electrophoresis, and then excised and eluted from the gel matrix with a QIAquick Gel Extraction Kit (Qiagen). The purified gut and fat body EH cDNA fragments then were random prime (Megaprime kit, Amersham), double-labeled with ^{32}P-dATP and ^{32}P-dTTP (Amersham) for use as gene-specific probes in Southern and Northern blots.

Determination of Probe Specificity

The full-length TmEH-1 and TmEH-2 EH cDNAs were excised from their respective plasmids by *Sac* I/*Sma* I (Promega) RE digestion. Digestion reactions included 0.7 μg plasmid (pG6-1 or pGUT-EH), 10 units of each RE and 1 x Buffer J (Promega). *Sma* I was added first for a 1 h incubation at 25^{o}C, and then *Sac* I was added to each reaction for an additional incubation for 1 h at 37^{o}C. A fraction of each reaction then was analyzed by agarose gel (0.7%) electrophoresis and ethidium bromide staining to verify the liberation of EH inserts of the appropriate size. For Southern blots, 6 ng of each digested plasmid were loaded in separate lanes of 0.7% agarose gels for electrophoresis. Gels were then denatured, neutralized and transferred to Hybond-N^{+} nylon membranes according to Sambrook *et al*., 1989. Nylon membranes were UV crosslinked in a Bio-Rad GS Genelinker after transfer. The membranes were pre-hybridized at 50^{o}C for 30 min in Rapid-Hyb buffer and then hybridized at 50^{o}C for 2.5 h with the TmEH-$1_{(205)}$ or TmEH-$2_{(327)}$ probe. Also at 50^{o}C, the membranes were washed twice in 2 x SSC, 0.2% SDS for 10 min per wash and then twice in 0.2 x SSC, 0.2% SDS for 15 min per wash. The blots were analyzed by autoradiography.

Northern Blot Analysis

Tissue samples were collected by dissection of *T. ni* larvae at L5D0 and on each day of the fifth stadium. Guts were prepared in the same manner as those for cDNA library construction. After removal of the gut, malpighian tubules were removed from the carcass and discarded. The fat body, which included tracheae and connective tissues, was then collected by scraping the integument with forceps. Total RNA was isolated from each sample of gut and fat body with an RNeasy Mini Kit (Qiagen). Ten micrograms of each RNA sample were separated by formaldehyde denaturing gel (1.25%) electrophoresis and transferred to Hybond-N^+ nylon membranes according to Sambrook *et a*l. (1989). Nylon membranes were cross-linked in a Bio-Rad GS Genelinker after transfer. The membranes were pre-hybridized at 55^{o}C for 30 min in Rapid-Hyb buffer and then hybridized at 55^{o}C for 2.5 h with the gene-specific probes, TmEH-$1_{(205)}$ or TmEH-$2_{(327)}$. Also at 55^{o}C, the membranes were washed twice in 2 x SSC, 0.2% SDS for 10 min per wash and then twice in 0.2 x SSC, 0.2% SDS for 15 min per wash. After analysis by autoradiography, the nylon membranes were stripped by washing two times (1 h per wash) in a boiling solution of 0.1 x SSC, 0.1% SDS (Sambrook et al., 1989). To evaluate the amount of total RNA loaded into each lane, the blots were re-hybridized at 55^{o}C for 2.5 h with a ribosomal RNA probe labeled with ^{32}P-dTTP using Redi-Prime (Amersham). Wash conditions were identical to those described above. This probe (a gift from Dr. Brian Wiegmann, Department of Entomology, North Carolina State University) was obtained by PCR amplification of 18S rRNA from *Coelopa frigida*.

TmEH-1 Baculovirus Expression In Vivo in T. ni Larvae

The TmEH-1 cDNA was subcloned into the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) vector system. The pG6-1 plasmid was sequentially digested with *Bam*H I (Promega) and *Xho* I (Stratagene), providing a fragment containing the EH message. This fragment (1908 bp) was ligated using T4 DNA ligase (Novagen, Madison, WI) with the viral transfer vector pBacPak8 (pBP8; CLONTECH, Palo Alto, CA), previously treated with *Bam*H I (Promega) and *Xho* I (Stratagene), which provided the recombinant baculovirus plasmid (pBP8-G6-1). Recombinant baculovirus was obtained by addition of pBP8-G6-1 (containing TmEH-1) and *Bsu* 361-digested BacPak6 viral DNA (CLONTECH), producing the non-occluded virus, vG6-1. All virus manipulations were according to O'Reilly et al. (1992). Two microliters of complete medium containing 3.0 x 10^5 pfu of either vG6-1 EH baculovirus or wild-type (AcMNPV; non-transformed) control baculovirus were injected under a proleg (second set from anterior) of L5D0 *T. ni*. Unifected control insects were injected with 2 μl of complete medium containing no baculovirus. Needles for injections were made from glass Pasteur pipettes that were melted and pulled for a smaller gauge opening and a sharper tip. The injection volume was aspirated and delivered by mouth inhalation and exhalation through a rubber hose attached to the base of the pipette needle. Separate needles and rubber hoses were used for each treatment. Larvae that bled excessively following injections were discarded. Insects were then placed on artificial diet and held at normal rearing conditions. The physical condition of treated larvae was observed every 24 h for 4 d.

RESULTS AND DISCUSSION

Nucleotide and Deduced Amino Acid Sequences of T. ni EH cDNA

Previously in our laboratory, a full-length EH cDNA (TmEH-1; NCBI access. #U73680) was cloned from a library made with the fat body of *T. ni* (VanHook Harris et al., 1999). This cDNA library was constructed with L5D3 larvae, which is the stage during the last stadium when fat body JH III *in vitro* EH activity is highest (Kallapur et al., 1996). In midgut, however, EH activity peaked on L5D2. The developmental differences in the levels of EH activity between fat body and midgut, and the results of Southern blots with *T. ni* genomic DNA (VanHook Harris et al., 1999) discussed previously, suggested that multiple EH genes may be present in *T. ni* larvae. As mentioned earlier, recent reports have shown the presence of at least two insect EH cDNAs in *C. felis* and *D. melanogaster*. In the present study, a cDNA library was contructed from the whole guts of an equal proportion of L5D1 and L5D2 *T. ni*, and then screened by PCR and Southern blotting with the $TmEH_{(237)}$ fragment (NCBI access. #U35736) as a probe. $TmEH_{(237)}$ is a 237 bp putative EH fragment generated by RT-PCR of total RNA from whole body L5D3 *T. ni* (VanHook Harris et al., 1999). An EH cDNA, called TmEH-2 (NCBI access. #AF035482), was subsequently cloned and sequenced from the *T. ni* gut library (Fig. 1.1). This cDNA contained the complete coding sequence of an EH protein. TmEH-2 and the $TmEH_{(237)}$ probe were 81% identical, and the region of overlap between these two sequences is single-underlined in Figure 1.1. The length of the TmEH-2 insert was 2054 bp, with 5'- and 3'-untranslated regions consisting of 29 and 636 bp, repectively. The putative polyadenylation signal, ATTAAA, began at nucleotide 1966 (double underlined, Fig. 1.1), while the poly-A tract started at nucleotide

1983. It is unusual that the polyadenylation signal was not AATAAA. This typical sequence is highly conserved but occasionally a single base may be different (Lewin, 1994), as was the case with TmEH-2. Overall, the sequence of TmEH-2 differed from TmEH-1, which was 1887 bp with 81 and 416 bp in the 5'- and 3'-untranslated regions, respectively. Unlike TmEH-2, TmEH-1 had the typical AATAAA polyadenylation signal. $TmEH_{(237)}$ also was used to isolate TmEH-1. The percentage identity of the probe to TmEH-1 was higher (94%) than to TmEH-2. The two EHs appear to be unique based on these variations and the percentage identities from sequence alignments. The nucleotide sequences of TmEH-1 and TmEH-2 were 67% identical, while the two EHs shared 73% identity of deduced amino acid sequences (GAP alignment analyses, SEQWeb; Appendix A). Despite the disparities in sequence, both *T. ni* EH cDNAs had open reading frames of 1389 bp, which coded for identically sized (463 amino acids) putative proteins. These findings provide further evidence that multiple EHs are present in *T. ni*. The TmEH-1 and TmEH-2 are at least different alleles of the same gene, and more likely are the products of two separate genes. When compared to other EH sequences, the amino acid sequence of TmEH-2 was only 46% identical to that of another lepidopteran, *M. sexta*. The percentage identities ranged from 42 to 47% when TmEH-2 was compared to all other insect EH amino acid sequences known to date (see Table 1.1 for species). Interestingly, the percentage identities of *T. ni* TmEH-2 with other insect EHs were similar when compared to mEHs of mammals. For example, TmEH-2 was 42 and 40% identical to rat and human mEH. However, when GAP-aligned with soluble EHs (sEHs) of rat and human, TmEH-2 was only 18 and 19% identical. These alignments suggest that TmEH-2, like TmEH-1, is a microsomal enzyme.

Phylogenetic Tree Based on Multiple EH Sequence Alignment

In 1995, Beetham et al. proposed a molecular classification system that discriminated between mEHs and sEHs based on percentage identities of amino acid sequences. Because of the small number of EHs sequenced at the time, the gene tree in that study consisted of only eight amino acid sequences. Among those sequences were three mammalian mEHs, three mammalian sEHs and two plant sEHs. This report demonstrated that EHs were clearly separated into a mEH or sEH cluster in the tree. A more recent alignment and phylogenetic tree that included additional EH sequences and other members of the α/β hydrolase-fold enzymes also partitioned mEHs and sEHs into separate clusters (Arand et al., 1999a). In this tree, the first two insect EHs sequenced from *M. sexta* (Wojtasek and Prestwich, 1996) and *T. ni* (TmEH-1; VanHook Harris et al., 1999) clustered with the mEHs.

In the current study, a phylogenetic tree was generated that included several EH sequences analyzed by Beetham et al. (1995) and Arand et al. (1999a), as well as a number of EH sequences from various organisms that either were not available or not included in these earlier accounts. Complete scientific names and NCBI accession numbers of all sequences analyzed in this report are listed in Table 1.1. Among the sequences in our analysis were seven novel insect EHs, including TmEH-2. In all, 34 EH sequences representing organisms from four kingdoms were aligned (see Appendix A for CLUSTAL X alignment), and the unrooted tree (Fig. 1.2) was inferred using the neighbor-joining algorithm of CLUSTAL X (v. 1.64) software. Like the two EH trees reported earlier, our phylogenetic tree partitioned all EHs analyzed into mEH or sEH clusters. Among the mEHs, the high degree of homology between taxonomically related organisms was obvious by distinct groupings of the fungi, bacteria, nematode, mammals

and insects. Like all other insect EHs, TmEH-2 was classified by the tree as a microsomal enzyme. As expected, TmEH-2 and TmEH-1 were joined by a common node in the tree. Both of the *T. ni* EHs were related more closely to the EH of another lepidopteran, *M. sexta*, than to all other insect EHs.

Within the sEHs, the grouping of taxonomically related organisms also was distinguishable in the phylogenetic tree, with the exception of three bacterial sequences. Two of these sequences, *Streptomyces coelicolor* and *Agrobacterium tumefaciens*, were positioned between plant sEHs. The third, *Bacillus subtilis*, appeared to be more related to mammalian than to the other bacterial or plant sEHs. It is noteworthy that these bacterial peptide sequences were translated conceptually from genomic sequences, which may or may not deviate from the true translations of the gene products. Therefore, firm conclusions regarding these sequences and their alignments should be reserved until confirmations can be made with cDNA sequences. Nonetheless, inclusion of the translated genomic sequences allowed for predictions of bacterial mEH and sEH relationships with EHs from organisms of other kingdoms. A total of six EHs in the analyses originated from genomic sequences (identified by underlined accession numbers in Table 1.1). All other EH amino acid sequences (28 of 34) used in these alignments were from complete coding cDNAs.

Multiple Sequence Alignment and Discovery of Variations in the Catalytic Triad of mEHs

To date, all insect EHs sequenced are evidently microsomal enzymes. The mechanism of hydration for insect mEH appears to resemble that of mammalian mEHs (Linderman et al., 1995). In mammals, this mechanism is an S_N2 reaction involving an attack of the substrate at the least substituted oxirane carbon by the carboxylate of Asp^{226}.

This results in a covalently bound acyl-enzyme intermediate (Lacourciere and Armstrong, 1993; Hammock et al., 1994; Tzeng et al., 1996; Müller et al., 1997). The neighboring Trp^{227} is believed to activate the epoxide towards the nucleophilic attack (Tzeng et al., 1996; Wojtasek and Prestwich, 1996). The intermediate ester then is hydrolyzed to the ester carbonyl by a water molecule that is activated by a Glu^{404}/His^{431}, or Glu^{376} + Glu^{404}/His^{431} charge-relay system (Laughlin et al., 1998; Tzeng et al., 1998; Arand et al., 1999b). While there is strong evidence for the participation of the Asp^{226} and His^{431} in the mechanism, there is some debate on whether Glu^{404} acts alone, or in conjunction with Glu^{376} as the partner(s) with His^{431} for the charge-relay system. Regardless of this dispute, it has been predicted that the partner(s) to His^{431} involves Glu for all mEHs. This is in contrast to the sEHs, which have a charge-relay system composed of Asp/His (Pinot et al., 1995; Arand et al., 1996). Although the catalytic triad has a second Asp, the hydration mechanism for sEHs (Borhan et al., 1995; Pinot et al., 1995; Arand et al., 1996) is similar to the mEHs.

The amino acid sequences of the 34 EHs in the phylogenetic tree were aligned (see Appendix A for CLUSTAL X alignment) and analyzed in the regions of the proposed catalytic triad (Fig. 1.3). As expected, the catalytic residues of TmEH-2 were identical to the those of TmEH-1 (Fig. 1.3A). Despite the earlier conception that all mEHs possess the Asp-Glu-His catalytic triad, our sequence alignments identified several mEHs that differed. In *D. melanogaster*, two mEHs (*D. melanogaster* II and III, NCBI access. #s AF517546 and AF517567, respectively) had the sEH-like catalytic residues, Asp-Asp-His (Fig. 1.3A). A third mEH (*D. melanogaster* I, NCBI access. # AF517545) from this insect had the expected Asp-Glu-His triad. All three full-length mEHs (NCBI submissions by Dr. Dov Borovsky et al., University of Florida, 2002) were cloned and

sequenced from a cDNA library consisting of gut, ovary and fat body from adult flies. Additionally, two of the bacterial mEHs also had the Asp-Asp-His triad (Fig. 1.3A). These EHs from *Streptomyces globisporus* and *Clostridium acetobutylicum* were translated conceptually from genomic sequences. As discussed earlier, caution should be heeded in the alignment analyses of these predicted peptide sequences. The other two bacterial mEH sequences in the alignment, *Stigmatella aurantiaca* and *Caulobacter crescentus*, exhibited the expected Asp-Glu-His triad. The Asp-Asp-His triad also was present in a mEH from *Aspergillus niger*. The presence of a second Asp in the catalytic triad of this fungus already has been reported by Arand et al. (1999a). The *A. niger* EH, however, is unique from the *D. melanogaster* and bacterial mEHs that had the Asp-Asp-His triad. Loosely defined, the *A. niger* EH seems to be a hybrid of microsomal and soluble forms. Intriguingly, this EH lacked the common N-terminal membrane anchor of mEHs, which rendered it soluble in the native environment. Despite its soluble nature and sEH-like catalytic triad, the *A. niger* EH was classified as microsomal by having greater overall sequence similarity to mEHs as compared to sEHs of other organisms.

The presence of the Asp in the charge-relay system of the *A. niger* EH, in contrast to a Glu residue in the respective position of other mEHs, may be one important contributor to an exceptionally high substrate turnover number of the fungal enzyme when compared to its mammalian relatives (Arand et al., 1999a). Two pieces of evidence support this hypothesis. First, when the catalytic Asp^{348} of *A. niger* EH was substituted with Glu, the expressed mutant clone retained catalytic activity against 4-nitrostyrene oxide, but the V_{max} and K_m were reduced significantly (Arand et al., 1999a). Second, replacing the typical mEH charge-relay Glu of rat mEH with Asp greatly increased the V_{max} and K_m for two structurally different epoxide substrates (Arand et al., 1999b).

Based on these results, it is possible that the *D. melanogaster* and bacterial mEHs having the Asp-Asp-His triad may also have enhanced catalytic activity. If this is the case, the rapid mEHs may be vital for metabolic events that differ from mEHs of mammals and even other insects or bacteria. One possible function of these mEHs in *D. melanogaster* is the metabolism of JH in both larvae and adults. In particular, the rapid mEHs may be necessary to efficiently metabolize JH III bis-epoxide. This homolog of JH has been discovered only in Diptera (de Kort and Granger, 1996), including *D. melanogaster* (Richard et al., 1989). Casas et al. (1991) have determined in metabolism studies that multiple EH isozymes may be present in order to metabolize JH III and JH III bis-epoxide in *D. melanogaster*. At present, however, a definitive purpose is unknown for this variant residue in the mEH catalytic triads of *D. melanogaste*r, *A. niger* or the two bacterial mEHs. As mentioned before, *D. melanogaster* also had one mEH with the expected Asp-Glu-His catalytic triad. It would be of great interest to heterologously express the two types of mEHs from this insect to compare enzyme kinetics with various substrates such as JH III bis-epoxide, other JH homologs and xenobiotic epoxides. These studies, along with experiments that analyze the enzyme activity after mutating the acidic charge-relay residues, could shed light on the importance of the variant catalytic triad in *D. melanogaster*.

Prior to this report, it was predicted that all membrane-anchored mEHs utilized Glu, while sEHs employed an Asp as the charge-relay partner to His. In fact, the presence of Glu or Asp in this position has been used as one criterion for determining if an EH is microsomal or soluble in origin. Although all sEHs had the Asp-Asp-His triad (Fig. 1.3B), the Asp-Asp-His variation in some mEH triads now makes this criterion ambiguous. EHs should be analyzed for relatedness to known mEHs or sEHs based on

several other criteria. Among these criteria are the presence or absence of an N-terminal membrane anchor sequence, sequence conservation among motifs that are specific to mEHs or sEHs, and location of the sequence in mEH or sEH clusters of a properly constructed phylogenetic tree that is inferred from a sound alignment of amino acid sequences.

Lastly, recall from above that reports on mammalian mEH were conflicting about whether Glu^{404} acts alone or with Glu^{376} in the charge-relay system with His^{431}. If both Glu residues are involved in mammalian and other mEHs, it now is pondered whether the *D. melanogaster* Glu^{384} (respective to the mammalian Glu^{376}) would assist, or perhaps even replace the Asp^{412} (respective to mammalian Glu^{404}) in partnering with His. The experiments proposed earlier involving the mutation of active site residues also could provide clues on the number and precise identity of charge-relay partner(s) with His in the mEHs of *D. melanogaster*.

Design and Evaluation of T. ni EH Gene-Specific Probes

The discovery of a full-length EH cDNA in this report validated the presence of two EHs in *T. ni* – TmEH-1 from fat body and TmEH-2 from the gut. Although each EH was isolated from a different tissue, it was unknown if each was tissue-specific. To determine the tissue specificity of TmEH-1 and TmEH-2 expression, DNA probes for Northern blots were designed from each cDNA. Preliminary tests using the full-length EH cDNAs as probes and samples failed due to cross-hybridization of TmEH-1 with TmEH-2, and vice versa, on Southern blots under high stringency conditions. Therefore, shorter probes were designed by using restriction endonucleases to digest the EH cDNAs within the 3'-untranslated regions, where the percentage identity between each was

significantly lower than the coding regions. As shown in the alignment in Figure 1.4, a 205 bp fragment (TmEH-$1_{(205)}$) was isolated from TmEH-1, and a 327 bp fragment (TmEH-$2_{(327)}$) was liberated from TmEH-2. The latter probe also is shown (blue text) within the full-length sequence of TmEH-2 in Fig. 1.1. While the identity between the two full-length cDNAs was 67%, the identity of each probe to the opposite full-length cDNA was lower. TmEH-$1_{(205)}$ was 48% identical to TmEH-2, while TmEH-$2_{(327)}$ was 41% identical to TmEH-1. Before use in Northern blots, each probe was tested for hybridization against both full-length EH cDNAs that were blotted onto nylon membrane. As shown in these Southern blots (Fig. 1.5A), TmEH-$1_{(205)}$ hybridized to the TmEH-1, but not to the TmEH-2 cDNA. Conversely, TmEH-$2_{(327)}$ hybridized to the cDNA of TmEH-2, but not to that of TmEH-1 (Fig. 1.5B). Based on these results, the probes were gene-specific for each EH within the limits of detection.

Expression Profiles of TmEH-1 vs. TmEH-2 in Fat Body and Gut of T. ni

To determine the tissue- and age-specificity of TmEH-1 and TmEH-2 expression, total RNA was isolated from *T. ni* fat body and gut on each day of the fifth stadium. The RNA samples then were separated by gel electrophoresis and Northern blotted. Blots were hybridized with the TmEH-$1_{(205)}$ or TmEH-$2_{(327)}$ gene-specific probes. Hybridization conditions for Northern blots were similar to the Southern blots for probe specificity tests, except that the temperature was raised 5^{o}C to account for the increased stability of a RNA:DNA hybrid over the DNA:DNA hybrid. As displayed in Figure 1.6, TmEH-$1_{(205)}$ was hybridized with two blots – one with fat body total RNA and the other with total RNA from gut. The detected signal in both blots suggested that TmEH-1, originally isolated from fat body, was expressed not only in fat body, but also gut. In

fact, the signal with the TmEH-$1_{(205)}$ probe was stronger in gut than in fat body. Although TmEH-1 was not tissue-specific, its expression was age-specific. In both tissues, expression was detectable at each day of the fifth stadium, but was highest on L5D0. The signal then diminished during each subsequent day of the fifth stadium, with the lowest expression levels in prepupae (L5D4).

When the TmEH-$2_{(327)}$ probe was hybridized to Northern blots containing total RNA from fat body or gut (Fig. 1.7), results were similar to those obtained with the TmEH-$1_{(205)}$ probe (Fig. 1.6). Apparently the expression of TmEH-2, isolated originally from gut, also lacked tissue-specificity. TmEH-$2_{(327)}$ hybridized to RNA from both tissues, and like TmEH-1, the signal for TmEH-2 was more intense in gut than in fat body RNA. Moreover, the age-specificity of TmEH-2 expression was analogous to that of TmEH-1. Expression was at the highest level on L5D0. The signal decreased throughout L5D1-L5D4, with the lowest level on L5D4. Approximately equal loading of total RNA was verified by stripping and hybridizing all Northern blots with a rRNA probe (Fig. 1.6 and 1.7).

The former report by VanHook Harris et al. (1999) on the discovery of TmEH-1 also evaluated EH expression in a Northern blot with total RNA from fat body. In that study, however, the probe used for hybridization was $TmEH_{(237)}$. As discussed earlier, this was the probe used in screening cDNA libraries to isolate TmEH-1 from fat body, and TmEH-2 from gut. The results of hybridization of fat body RNA with $TmEH_{(237)}$ differed markedly from our studies using the gene-specific probes, TmEH-$1_{(205)}$ and TmEH-$2_{(327)}$. Instead of a diminishing signal from L5D0 through L5D4, the signal using $TmEH_{(237)}$ was low on L5D0, increased on L5D1, and peaked on L5D2. EH expression then decreased dramatically on L5D3, and moreso on L5D4 (VanHook Harris et al.,

1999). The variations in fat body EH expression with $TmEH_{(237)}$ vs. $TmEH\text{-}1_{(205)}$ and $TmEH\text{-}2_{(327)}$ are not fully understood. The $TmEH_{(237)}$ probe was a PCR product from whole body *T. ni* RNA, using degenerate primers designed from a consensus sequence surrounding the active site of EHs from a variety of organisms. It is internal to the coding sequence where TmEH-1 and TmEH-2 share high homology. As a consequence, the $TmEH_{(237)}$ probe likely detected all EH expression in *T. ni*. This potential cross-hybridization was unlikely using the gene-specific probes, $TmEH\text{-}1_{(205)}$ and $TmEH\text{-}2_{(327)}$.

Baculovirus Expression of TmEH-1 In Vivo in T. ni Larvae

The involvement of JH esterase in insect development has been demonstrated in Lepidoptera with selective *in vivo* inhibitors of the enzyme (Roe et al., 1997), and by the effects on larvae infected with a JH esterase-transformed baculovirus (Hammock et al., 1990). Injection of the JH esterase baculovirus into second stadium *M. sexta* led to blackening of the larvae, while feeding it to first stadium larvae reduced weight and growth when compared to control virus treatments. Both of these conditions likely are due to an untimely decrease in JH titer caused by the increase in JH esterase levels from viral expression (Hammock et al., 1990). The increase in JH esterase levels compared to control treatments was validated by measuring activity *in vitro* in hemolymph of infected insects. As discussed earlier, the importance of EH in decreasing JH titer for the initiation of metamorphosis still is unknown. A major reason for this uncertainty is because no potent *in vivo* inhibitors of insect EH have been discovered to allow developmental observations in the absence of enzyme activity. Therefore, in an attempt to shed light on the importance of EH in larval development, the TmEH-1 cDNA was subcloned into a baculovirus system and expressed *in vivo* in *T. ni* larvae. Only TmEH-1

was subcloned for expression since there was a high percentage of identity between this EH and TmEH-2, and because both EHs were expressed in fat body and gut. Also, a previous study already has shown TmEH-1 to be JH-specific when compared to other epoxide substrates *in vitro* after expression in *Spodoptera frugiperda* (Sf9) cells (VanHook Harris, 1999). If TmEH-1 plays a role in clearing JH *in vivo*, its overexpression by the transformed baculovirus could cause an untimely decrease in JH titer. Such a decrease may elicit signs of delayed development in larvae. Larvae in this experiment were treated by injecting L5D0 *T. ni* with the TmEH-1-transformed, or non-transformed control (wild-type) baculovirus. When equal titers of each non-occluded baculovirus were injected, there were no differences in physical appearance between insects of each treatment during the course of the experiment. Furthermore, the TmEH-1 virus caused no significant reduction in body weight during the course of the experiment (data not shown). Surprisingly, however, the lethal time of TmEH-1 baculovirus was shorter than the control virus. While no mortality occurred at 24 h post-injection for any treatment, the TmEH-1 baculovirus elicited 48% cumulative mortality at 48 h, compared to 29% for the control baculovirus treatment. The difference between treatments was greater (2.3-fold) and statistically significant (t-test, $\alpha = 0.05$) at 72 h post-injection, as cumulative mortality was 95 and 41% for the TmEH-1 and control baculoviruses, respectively. By 96 h after injection, both treatments resulted in 100% mortality. The cumulative mortality of uninfected control treatments (injections with media that lacked baculovirus) did not exceed 15%. The results of the TmEH-1 baculovirus were similar to lethal times of a scorpion peptide neurotoxin-transformed baculovirus that was injected into *Bombyx mori* (silkworm; Maeda et al., 1991). In that study, insects died between 60-75 h after injection. The mechanism of accelerated toxicity of the EH-transformed

baculovirus in *T. ni* is not known at this time. The enhanced virulence may not involve a decrease in JH titer, since there was no increase in whole body JH III EH activity *in vitro* when compared to control baculovirus treatments (data not shown). TmEH-1 did appear to have been expressed *in vivo*, however, based on SDS-PAGE analysis. TmEH-1 baculovirus-treated larvae had higher levels of protein at the expected size for this EH than insects treated with control virus (data not shown). TmEH-1 activity might have been degraded rapidly by proteolytic enzymes in the infected insects. The virally induced production of ecdysteroid UDP-glucosyl transferase could have reduced EH activity, as was predicted with low JH esterase baculovirus expression *in vivo* (Hammock et al., 1990). Based on these observations, it is possible that the mechanism of enhanced toxicity is non-specific. Because TmEH-1 is hydrophobic and membrane-bound, the overexpression of this protein could interfere with normal cellular membrane functions even if the enzyme has been deactivated. This non-specific mechanism has been suggested previously as the cause of enhanced toxicity for a baculovirus transformed with URF13, a membrane-bound maize mitochondrial protein (Korth and Levings, 1993). The URF13 baculovirus killed all *T. ni* larvae significantly faster (within 60 h) than the non-transformed control virus after injection of the non-occluded forms.

Regardless of the mechanism of increased toxicity, the lack of enhanced EH activity *in vivo* by the TmEH-1 baculovirus prevented any conclusions on whether or not EH has a significant role in titer regulation of JH. Nonetheless, the fact that the lethal time of the baculovirus was decreased significantly by inclusion of an insect EH is intriguing in itself. The potential of using genetically engineered baculoviruses for insect control has been studied thoroughly (see reviews by: Possee and Bishop, 1992; Bonning and Hammock, 1996; Possee et al., 1997). However, in recent years the interest in

developing these baculoviruses as pesticides has waned mainly due to their slow speed of action compared to conventional insecticides, and problems with stability under field conditions. If problems are resolved in the future for use of genetically engineered baculoviruses as pesticides, it would be of interest to study the TmEH-1 baculovirus further, including the evaluation of lethal time upon oral adminstration in the occluded form. Additionally, an understanding of the mechanism of accelerated toxicity could be valuable in the optimization of the TmEH-1-transformed baculovirus as an effective biopesticide.

Summary

The cloning of a second, unique EH cDNA (TmEH-2) from *T. ni* has provided further evidence for the existence of multiple EH alleles or genes in this species. The RNAs from the two *T. ni* EHs appear to be regulated similarly, as their expression was age-specific, but not tissue-specific. Contrary to previous conceptions, not all membrane-anchored mEHs contain Asp-Glu-His as the catalytic triad. Several mEHs were discovered that possessed the sEH-like triad of Asp-Asp-His. Consequently, the identification of catalytic residues no longer can serve as one of the discriminating criteria between mEHs and sEHs. Determination of the relatedness of an EH cDNA to microsomal or soluble EHs of other organisms is best accomplished in a phylogenetic tree inferred from amino acid sequence alignments. Based on the tree in this report, both *T. ni* EHs, like all other insect EHs, were classified as microsomal. Lastly, *in vivo* baculovirus expression of TmEH-1 in *T. ni* larvae did not shed light on the role of EH in JH metabolism. However, the TmEH-1-transformed baculovirus accelerated toxicity significantly in fifth stadium *T. ni*, as compared to the non-transformed control virus.

Further studies are needed to elucidate the mechanism behind this enhanced virulence of the TmEH-1 baculovirus.

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Figure 1.1. Nucleotide and deduced amino acid sequences of the full-length epoxide hydrolase cDNA (TmEH-2; NCBI access. #AF035482) cloned from the gut RNA of equal proportions of L5D1 and L5D2 *T. ni*. Untranslated regions are in lower case type. The region of overlap between TmEH-2 and the EH probe ($TmEH_{(237)}$; NCBI access. #U35736) used for cDNA library screening is single-underlined. The proposed amino acid residues of the catalytic triad are in colored text: D, red; E, green; H, purple. The portion used as a gene-specific probe ($TmEH\text{-}2_{(327)}$; also see Fig. 1.4) is in blue text. The putative polyadenylation signal is double-underlined.

```
   1                                                                                                caacttttttatttcaattgtgtttttaat

  30  ATGGCCCGTCTCCTCTTCATACTACCAGTATTGGCACTGGTCTTTCTCCCAGTATACTTCTTATTCCTACAAAGTCCTCCA
      M  A  R  L  L  F  I  L  P  V  L  A  L  V  F  L  P  V  Y  F  L  F  L  Q  S  P  P

 111  CCGGTACCCAATGTTGACATGAACGATTGGTGGGGGCCTGAGAGCGCGAAAGAAAAACAGGATACCAGTATCAGGCCATTC
      P  V  P  N  V  D  M  N  D  W  W  G  P  E  S  A  K  E  K  Q  D  T  S  I  R  P  F

 192  AAAATCAGTTTTGGAAATAATAACGTCAAAGACTTAAAAGATCGTCTCCAAAGAACAAGACCGCTAACACCTCCACTAGAA
      K  I  S  F  G  N  N  N  V  K  D  L  K  D  R  L  Q  R  T  R  P  L  T  P  P  L  E

 273  GGTGTCGGCTTCGACTACGGCTTCAACACCAACGAAATCGACAGCTGGTTGAAGTACTGGGCCAAGGACTACAATTTCAAG
      G  V  G  F  D  Y  G  F  N  T  N  E  I  D  S  W  L  K  Y  W  A  K  D  Y  N  F  K

 354  GAGAGGGAAACTTTCTTGAACCAGTTCCCGCAGTTCAAGACTAACATCCAGGGACTGGATATACACTTCATTAGGGTTACG
      E  R  E  T  F  L  N  Q  F  P  Q  F  K  T  N  I  Q  G  L  D  I  H  F  I  R  V  T

 435  CCAAAGGTTCCTCAAGGGGTTGAAGTTGTTCCCCTCCTTCTTCTCCACGGCTGGCCAGGATCCGTCAGGGAGTTCTATGAA
      P  K  V  P  Q  G  V  E  V  V  P  L  L  L  L  H  G  W  P  G  S  V  R  E  F  Y  E

 516  GCCATCCCTCTGCTGACTGCTGTCAGCAAGGACAGAGACTTTGCCTTTGAAGTCATCGTGCCCAGTCTTCCTGGTTATGGG
      A  I  P  L  L  T  A  V  S  K  D  R  D  F  A  F  E  V  I  V  P  S  L  P  G  Y  G

 597  TTCTCTGATCCGGCAGTACGACCAGGACTGGGAGCGCCTCAGATTGGCGTCGTGATGAAGAACCTGATGAGCAGGCTCGGA
      F  S  D  P  A  V  R  P  G  L  G  A  P  Q  I  G  V  V  M  K  N  L  M  S  R  L  G

 678  TACAAGCAGTTCTACCTGCAAGGCGGTGACTGGGGAGCACTCATTGGAAACTGTATCGTAACTCTATTCCCTAAGGATATC
      Y  K  Q  F  Y  L  Q  G  G  D  W  G  A  L  I  G  N  C  I  V  T  L  F  P  K  D  I

 759  CTTGGTTACCACACTAACATGCCCATCGTGATGTCAGCAAAATCCACCTTATTTGAACTCCTTGGTTCAGTCTTCCCATCT
      L  G  Y  H  T  N  M  P  I  V  M  S  A  K  S  T  L  F  E  L  L  G  S  V  F  P  S

 840  CTGATTCTGGAAGACATGAGTACCTATGAAAGGTTGTATCCTCTGTCAACGAGGTTTGCTAACCTGCTGCGGGAGACTGGC
      L  I  L  E  D  M  S  T  Y  E  R  L  Y  P  L  S  T  R  F  A  N  L  L  R  E  T  G

 921  TACATGCATATTCAATCGACGAAGCCTGATACTGTTGGAGTTGCACTTTCGGATTCCCCAGCGGGTCTTCTAGCCTACATC
      Y  M  H  I  Q  S  T  K  P  D  T  V  G  V  A  L  S  D  S  P  A  G  L  L  A  Y  I

1002  CTCGAGAAGTTCGCAACCTGGACTCGGCCTGACCTGATGTCTAAACCCAATGGAGGTCTGGACTACCGCTTCACCAGAGAC
      L  E  K  F  A  T  W  T  R  P  D  L  M  S  K  P  N  G  G  L  D  Y  R  F  T  R  D

1083  CAGCTGATTGACAACCTAATGATGTACTGGACCAACAGAGCGATCACGCCGGCCATGAGATTGTATGCCGAGAACTTTAAT
      Q  L  I  D  N  L  M  M  Y  W  T  N  R  A  I  T  P  A  M  R  L  Y  A  E  N  F  N

1164  AAGAGGACTGTGGAAATGAAACTTGATGAGATCCCAACTCCAGTGCCAACATGGGGCTTGCAAACGAAATACGAATTAGGA
      K  R  T  V  E  M  K  L  D  E  I  P  T  P  V  P  T  W  G  L  Q  T  K  Y  E  L  G

1245  TATCAACCTAAATATATCCTAAAAATTAAGTTCCCAAACCTAGTCGGCACCACAGTACTTCAAGAGGGAGGTCACTTCATT
      Y  Q  P  K  Y  I  L  K  I  K  F  P  N  L  V  G  T  T  V  L  Q  E  G  G  H  F  I

1326  GCCTTCGAATTGCCCGAGGTCTTTACGAATGACGTCATCAAGGCTGTGACAGAGTTCAGAAAACTGCAGAAGAAGAATGTC
      A  F  E  L  P  E  V  F  T  N  D  V  I  K  A  V  T  E  F  R  K  L  Q  K  K  N  V

1407  AAGACTGATTTGTAAttatttgattttgaaactggaaatgtattaatgtaattgttgacaaggaagtgaaggaagttgtta
      K  T  D  L  *

1488  ggataatgaagatgatatatggctcgtatttttttgtcaaataacgaaaatgatttttttttttgcatccctgactcctgtg

1569  ctcgggattaggacttcccagagtctgcgttaatagcccgaattcaaattaaaccgttacagcaaccgctaaatttcgtag

1650  aattttacgtcatcatacgagattttggagcaatgtaatttcgtctaccattaaaaaattaaataagatgtattaccagct

1731  atactcaattccctgtgctatgcatttctattattaaatagttaaacacgacagtagaaaaagaaaatatagtacaagggt

1812  gctgcttatttcaaaagaaatctcttccagaaaacccgctatagaaaagtggttattattattgggtacgcattcatcggg

1893  ttgtatttaatcgcagtcttgttgtgttgacaacatttacttttaaatatattatgcaacctgtttttttttgttattaaatt

1974  atcttacataaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
```

Table 1.1. Accession numbers of amino acid sequences used in the alignment of epoxide hydrolases

EPOXIDE HYDROLASES	NCBI SEQUENCE ACCESSION NUMBER[a]
MICROSOMAL	
Trichoplusia ni (cabbage looper), TmEH-2	AF035482
Trichoplusia ni, TmEH-1	U73680
Manduca sexta (tobacco hornworm)	U46682
Ctenocephalides felis I (cat flea)	AF503908
Ctenocephalides felis II	AF503909
Aedes aegypti (yellow fever mosquito)	AF517544
Drosophila melanogaster I (fruit fly)	AF517545
Drosophila melanogaster II	AF517546
Drosophila melanogaster III	AF517547
Homo sapiens (human)	J03518
Sus scrofa (pig)	AB000883
Oryctolagus cuniculus (rabbit)	M21496
Rattus norvegicus (rat)	M26125
Mus musculus (mouse)	NM_010145
Caenorhabditis elegans	NM_072249
Rhodosporidium toruloides	AF416992
Rhodotorula glutinis	AF172998
Xanthophyllomyces dendrorhous	AF166258
Aspergillus niger	AJ238460
Stigmatella aurantiaca	AJ276219
Caulobacter crescentus	AAK25053
Streptomyces globisporus	AAL06662
Clostridium acetobutylicum	AAK78696
SOLUBLE	
Homo sapiens	L05779
Rattus norvegicus	X65083
Mus musculus	L05781
Solanum tuberosum (potato)	U02494
Arabidopsis thaliana (mouse-eared cress)	D16628
Glycine max (soybean)	D63781
Nicotiana tabacum (tobacco)	U57350
Ananas comosus (pineapple)	AY098527
Streptomyces coelicolor	T36559
Bacillus subtilis	F69801
Agrobacterium tumefaciens	AAK87581

[a]Accession numbers not underlined are from the NCBI Nucleotide database, and their sequences were derived from computational translation of cDNA. Underlined accession numbers are from the NCBI Protein database. These amino acid sequences were derived by the submitters from conceptual translation of genomic sequences.

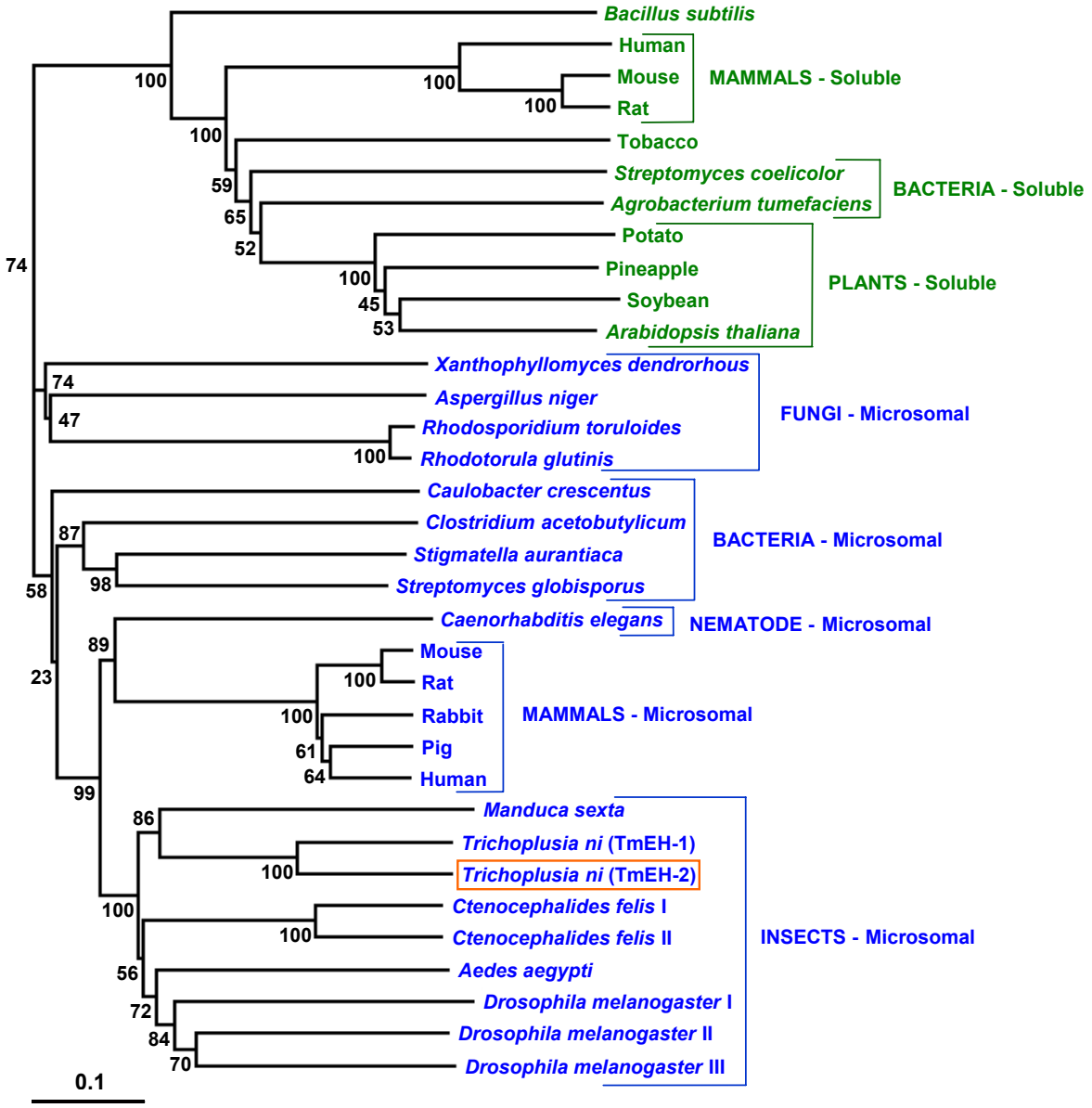

Figure 1.2. Phylogenetic tree of a selection of epoxide hydrolase enzymes from organisms spanning four kingdoms. The unrooted tree was inferred using the neighbor-joining method with uncorrected pairwise distances among amino acid sequences aligned in CLUSTAL X version 1.64b (see Appendix A). Sequences were obtained from NCBI Databases (see Table 1.1 for accession numbers). Epoxide hydrolases from organisms in green text are from the soluble cell fraction, while those in blue text are microsomal in origin. The numbers on the branches are bootstrap confidence levels for 100 pseudo-replicated data matrices. The marker of 0.1 is the length that corresponds to a 10% sequence difference. TmEH-2 (from *T. ni* gut, Fig. 1.1) is highlighted with an orange border.

Figure 1.3. Comparison of the amino acid sequences of the epoxide hydrolase (TmEH-2, Fig. 1.1) from *T. ni* gut with sequences of a selection of known epoxide hydrolases (Table 1.1; Fig. 1.2) in the regions of the catalytic triad. Sequences were aligned (see Appendix A) using CLUSTAL X version 1.64b software. The proposed catalytic residues are in colored upper case letters. (A) Microsomal epoxide hydrolases. (B) Soluble epoxide hydrolases. When the microsomal and soluble epoxide hydrolase groups were analyzed separately, those positions having 100% identity of amino acids are marked with an asterisk (*). Positions marked with a tilde (~) within microsomal or soluble epoxide hydrolases have 91% identity among amino acids.

(A) MICROSOMAL

INSECTS		~**~*				~~** * *
T. ni TmEH-2	221	ylqggDwgal	399	qtkyElgyqp	428	ggHfiafelp
T. ni TmEH-1	221	fvqggDwgsv	399	qgkyEiayqp	428	ggHffafelp
M. sexta	222	yiqagDwgsq	397	nfkyEvlyqp	426	ggHfaalhtp
C. felis I	219	yvqggDwgsm	396	kfpnElayvt	425	ggHfaafeep
C. felis II	219	yvqggDwgsr	396	kfphEiaykt	425	ggHfaaleep
A. aegypti	227	yvhggDwgsv	404	kfryElfqqt	432	ggHfvamqlp
D. melanogaster I	231	lvqggDwgsi	408	rfahEithfs	437	ggHfpafelp
D. melanogaster II	231	fiqggDwgsi	408	rfrfDlasvt	437	gsHfaalemp
D. melanogaster III	236	fiqggDwgsi	413	rfksDimqfl	442	ggHfaalevp
MAMMALS						
Human	221	yiqggDwgsl	400	afpfEllhtp	429	ggHfaafeep
Pig	221	ylqggDwgsl	399	afpsEvlhcp	428	ggHfaafeep
Rabbit	221	yiqggDwgal	400	afpcEimhvp	429	ggHfaafeep
Rat	221	yiqggDwgsl	400	afpsEllhap	429	ggHfaafeep
Mouse	221	yiqggDwgsl	400	afpsEilhap	429	ggHfaafeep
NEMATODE						
C. elegans	222	ylqggDwgai	400	sglnElydrt	428	mgHfaafeap
FUNGI						
R. toruloides	185	maqggDwgsi	355	lypgEiycpa	383	ggHfaalekp
R. glutinis	185	maqggDwgsi	355	lypgEiycpa	383	ggHfaalekp
X. dendrorhous	176	tcgagDwgsw	355	ifpaEitqyp	383	ggHfaavdnp
A. niger	187	iiqggDigsf	344	ffpkDlcpvp	372	ggHfaalerp
BACTERIA						
S. aurantiaca	232	vaqggDwgag	375	vfprElfrap	404	ggHfaafeqp
C. crescentus	171	laqggDwggl	326	hfpaEillpp	356	ggHfaameep
S. globisporus	170	vaqggDwgkv	332	vfpdDatrpi	361	ggHfaameqp
C. acetobutylicum	173	aaaggDvgsq	329	ifpaDillpp	357	ggHftameep

(B) SOLUBLE

MAMMALS		~*~		*		*
Human	329	vfighDwggm	491	taekDfvlvp	521	cgHwtqmdkp
Rat	328	vfighDwagv	491	taekDivlrp	521	cgHwtqiekp
Mouse	328	vfighDwagv	491	taekDivlrp	521	cgHwtqiekp
PLANTS						
Potato	100	fvvahDwgal	261	vgefDlvyhi	298	saHfvnqerp
A. thaliana	98	fvvghDwgal	261	igelDlvyym	298	vaHfinqekp
Soybean	121	flvahDwgai	281	tgelDmvyns	318	vaHfnnqeaa
Tobacco	96	flvgkDfgar	251	mgekDyalkf	287	gsHfvqeqfp
Pineapple	99	fvvghDwgaa	258	vgdlDltyhy	295	vgHfiqqera
BACTERIA						
S. coelicolor	121	vvvghDwgan	296	ggalDasttw	329	cgHwlqqerp
B. subtilis	98	ivighDwgga	233	wgmeDrflsr	263	asHwinhekp
A. tumefaciens	121	vvvghDwgat	293	igerDtglsi	328	agHwlqqerp

Figure 1.4. Design of epoxide hydrolase gene-specific probes from TmEH-1 (from *T. ni* fat body), and TmEH-2 (from *T. ni* gut, Fig. 1.1) cDNAs. Shown is a GAP alignment (GCG SEQWeb Sequence Analysis software, version 1.1) of the 3'-untranslated regions of the two sequences. The stop codons are in red text. Using *Hpa* I and *Nco* I restriction endonucleases, a 205 bp probe fragment (TmEH-$1_{(205)}$; purple text) was produced from TmEH-1. A 327 bp probe fragment (TmEH-$2_{(327)}$; blue text) was liberated with *Dra* I and *Eco*R I from TmEH-2. Following digestions, probes were separated and purified by gel electrophoresis and elution.

```
TmEH-2    AGAATGTCAAGACTGATTTGTAAttatttgattttgaaactggaaatgta 1448
          | || |  ||||||||||||| || |   | |     ||    || ||
TmEH-1    ATAACGAGAAGACTGATTTGTGAtgaggggttaggagaaaataaagtggt 1500


          ttaatgtaattgttgacaaggaagtgaaggaagttgttaggataatgaag 1498
          | | ||   |||||  |       |  |     | ||      ||
          tgactgattttgttagctttttctttaatttttggtctaccaaactttt 1550


          atgatatatggctcgtatttttttgtcaaataacgaaaatga.ttttttt 1547
             |   |||  | | |  ||| | | || | | |    | |    | ||
          tatagtaatgagttgcacgtttatattaagtcatgcgtttaagcagtctt 1600


          tttgcatccctgactcctgtgctcgggattaggacttcccagagtctgcg 1597
           ||||| || ||      |  || |     |   |   |||      | |
          CTTGCAACcatggtctgcgacctggtcgaaacttcaagccatcaaaagtg 1650
                  Nco I

                    EcoR I
          ttaatagcccgaattcaaattaaaccgttacagcaaccgctaaatttcgt 1647
           |    |   |  || |  ||  |     | |  |     |||  |  ||
          atgcatgtttgcgttaattttccatttagataatataatataagctaagt 1700


          agaattttacgtcatcatacgagattttggagcaatgtaatttcgtctac 1697
          | ||                            | | || |||     ||
          aaaa..........................ctaagttattcttaatat 1722


          cattaaaaaattaaataagatgtattaccagctatactcaattccctgtg 1747
           | |  | | |  ||  |   |||  | | | | ||     |      |
          aaatctagagtacaaataatcgtagaaactgatttaaattctaatacctc 1772


          ctatgcatttctattattaaatagttaaacacgacagtagaaaaagaaaa 1797
             || |  | | ||| |   ||    ||| | |   |   ||
          tattggaaatatcttaatcgttacaataacgccaacctgttaacctgttc 1822
                                                 Hpa I

          tatagtacaagggtgctgcttatttcaaaagaaatctcttccagaaaacc 1847
          || | ||   |      ||||  ||  ||| |||||  ||  | |
          tagactagctg......gctttcttgtaaataaatcaatttaatactctt 1866


          cgctatagaaaagtggttattattattgggtacgcattcatcgggttgta 1897
            | | | ||||      |
          gtcaaaaaaaaaaaaaaaaaa............................. 1887

                                             Dra I
          tttaatcgcagtcttgttgtgttgacaacatttactttaaatatattatg 1947
          caacctgtttttttgttattaaattatcttacataaaaaaaaaaaaaa 1997
          aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa 2047
          aaaaaaa                                            2054
```

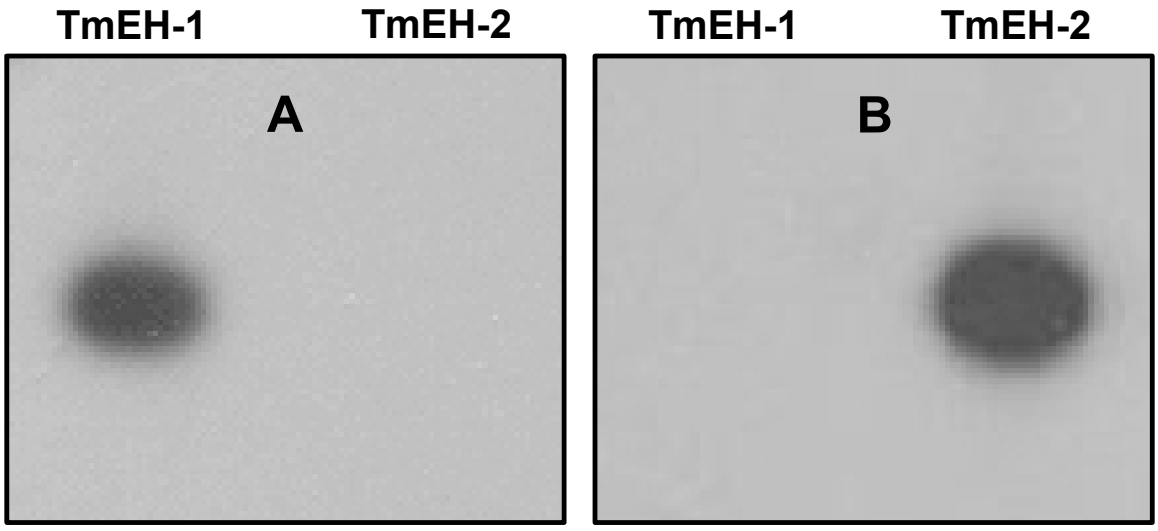

Figure 1.5. Determination of probe specificity. Plasmids (pG6-1 containing TmEH-1, and pGUT-EH containing TmEH-2) were digested to liberate the full-length *T. ni* EH cDNAs. Six ng of each digested plasmid were loaded in separate lanes of an agarose gel. After electrophoresis, the separated DNA samples were transferred to nylon membranes, denatured and hybridized with ^{32}P-labeled TmEH-1$_{(205)}$ (A) or TmEH-2$_{(327)}$ (B) probe. Washing conditions are described in Materials and Methods. These results were duplicated with different preparations of digested plasmids and probes.

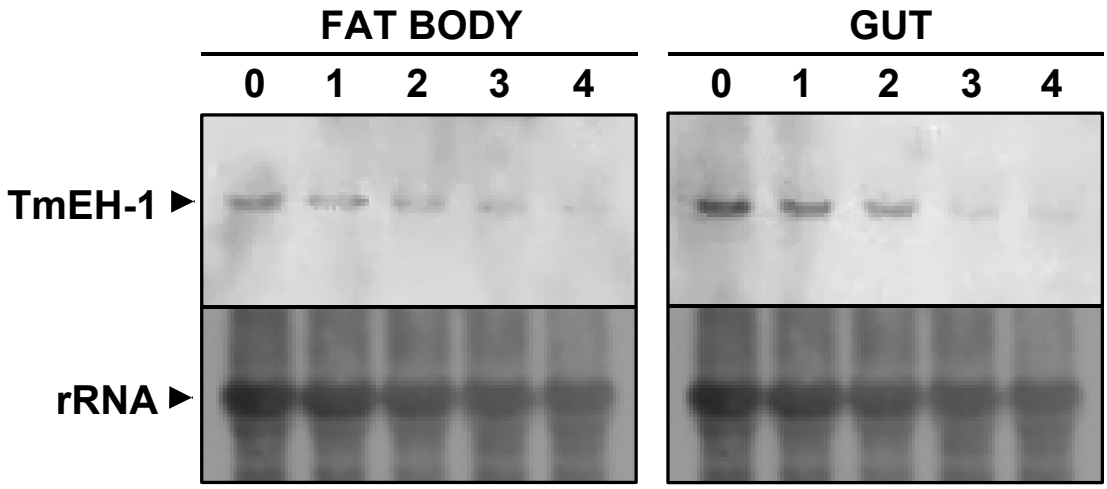

Figure 1.6. Northern blot analysis of total RNA from *T. ni* fat body (left panels) versus gut (right panels). The upper panels represent blots that were hybridized simultaneously with the gene-specific, ^{32}P-labeled TmEH-1$_{(205)}$ probe. Ten micrograms of total RNA from each day of the fifth stadium were loaded per lane. Numbers above lanes indicate the day of the fifth stadium. The probe originated from the full-length EH cDNA (TmEH-1) isolated from *T. ni* fat body. The signal visualized was approximately 2 kb in size. These blots were stripped and re-hybridized with a rRNA probe (bottom panels) to estimate loading quantities of RNA. Results shown were duplicated with different preparations of total RNA and probes.

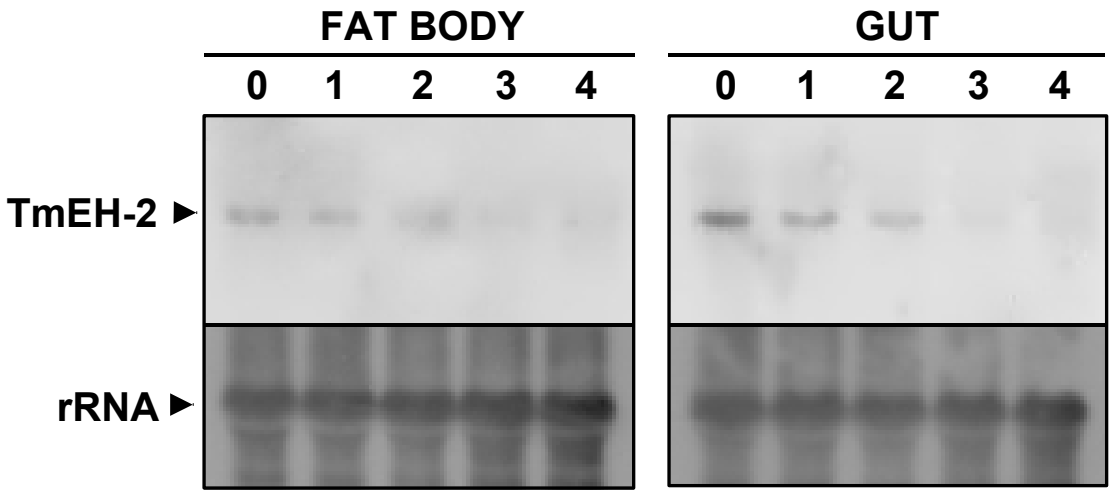

Figure 1.7. Northern blot analysis of total RNA from *T. ni* fat body (left panels) versus gut (right panels). The upper panels represent blots that were hybridized simultaneously with the gene-specific, ^{32}P-labeled TmEH-2$_{(327)}$ probe. Ten micrograms of total RNA from each day of the fifth stadium were loaded per lane. Numbers above lanes indicate the day of the fifth stadium. The probe originated from the full-length EH cDNA (TmEH-2) isolated from *T. ni* gut. The signal visualized was approximately 2 kb in size. These blots were stripped and re-hybridized with a rRNA probe (bottom panels) to estimate loading quantities of RNA. Results shown were duplicated with different preparations of total RNA and probes.

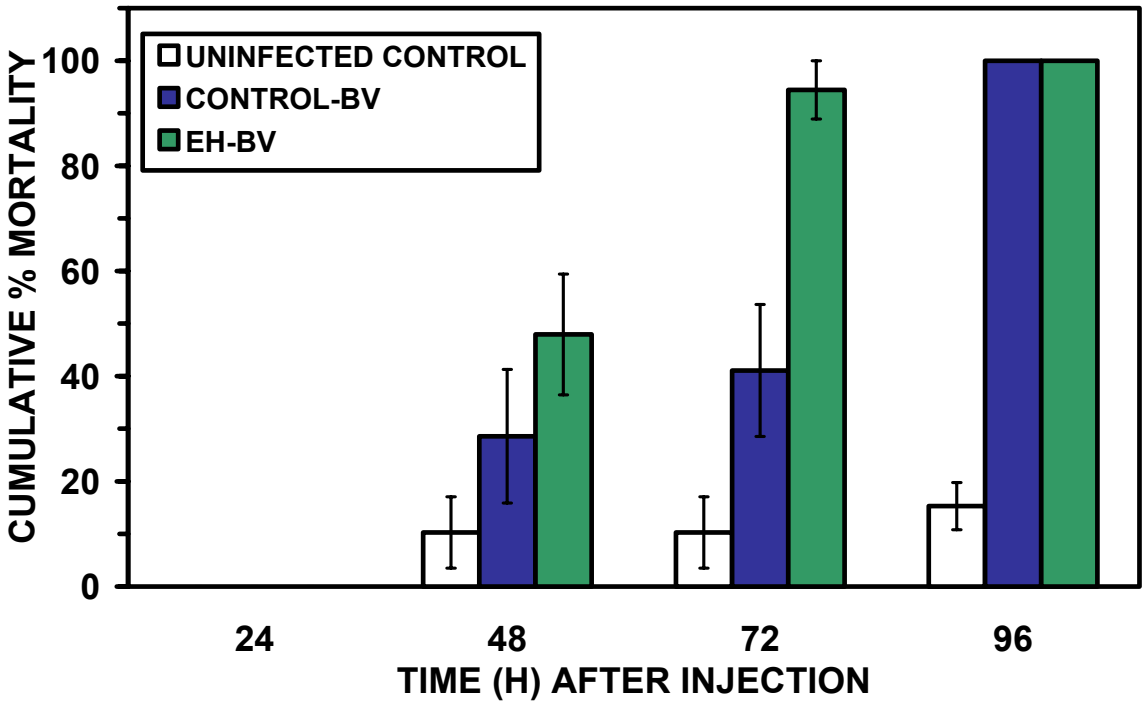

Figure 1.8. Effects of *in vivo* expression of TmEH-1 recombinant, non-occluded baculovirus in *T. ni* larvae. Insects were injected at L5D0 with 2 µl of complete medium containing 3.0 x 10^5 pfu of either EH-transformed baculovirus (EH-BV) or non-transformed, wild-type control baculovirus (Control-BV). Uninfected control larvae were injected with 2 µl of complete medium containing no baculovirus. Each value is the mean of four replicates (10 insects per replicate) and error bars represent $\pm$ 1 SEM.

CHAPTER 2

REGULATION OF JH EPOXIDE HYDROLASE VERSUS JH ESTERASE ACTIVITY IN THE CABBAGE LOOPER, *TRICHOPLUSIA NI*

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ABSTRACT

Juvenile hormone (JH) III esterase and JH III epoxide hydrolase (EH) *in vitro* activity was compared in whole body *Trichoplusia ni* homogenates at each stage of development (egg, larva, pupa and adult). While activity of both enzymes was detected at all ages tested, JH esterase was significantly higher than EH activity at all time points except day three of the fifth (last) stadium (L5D3). For both enzymes, activity was highest in eggs. Interestingly, adult virgin females had 4.6- and 4.0-fold higher JH esterase and EH activities, respectively, than adult virgin males. JH III metabolic activity also was measured in whole body homogenates of fifth stadium *T. ni* that were fed a nutritive diet (control) or starved on a non-nutritive diet of alphacel, agar and water. With larvae that were starved for 6, 28 and 52 h, EH activity per insect equivalent was 48, 5 and 1%, respectively, of the control insects. At the same time points, JH esterase levels in starved *T. ni* were 29, 4 and 3% of insects fed the nutritive diet. Selected insect hormones and xenobiotics were administered topically or orally to fifth stadium larvae for up to 52 h, and the effects on whole body EH and JH esterase activity were then analyzed. JH III induced JH III esterase as high as 2.2-fold, but not JH III EH activity. The JH analog, methoprene, increased both JH esterase and EH activity as high as 2.5-fold. The JH esterase inhibitor, 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP), had no impact on EH activity. The epoxides, *trans*- and *cis*-stilbene oxide (TSO and CSO) each induced EH between 1.9- and 2.0-fold. TSO did not alter JH esterase levels when topically applied, but oral adminstration reduced activity to 70% of the control at 28 h, and then induced activity 1.8-fold at 52 h after treatments began. CSO did not have an impact on JH esterase activity. Phenobarbital increased EH activity by 1.9-fold, but did

not change JH esterase levels. Clofibrate and cholesterol 5α,6α-epoxide had no effect on EH. JH esterase activity also was not affected by clofibrate, but cholesterol 5α,6α-epoxide was inhibitory to JH esterase, lowering activity to 60-80% of the control. The biological significance of these results is discussed.

INTRODUCTION

Embryogenesis, development, metamorphosis and reproduction of insects are controlled by the titer of juvenile hormone (JH). JH titer is regulated by a balance between the rate of its synthesis and degradation (reviewed by Gilbert et al., 2000). The primary routes of JH degradation are hydrolysis of the methyl ester by JH esterase and hydration of the 10,11-epoxide by epoxide hydrolase (EH), which yield the carboxylic acid and diol, respectively (Slade and Zibitt, 1972; for reviews, see Hammock, 1985; Roe and Venkatesh, 1990; de Kort and Granger, 1996). While JH esterase is found in hemolymph and other tissues, insect EH is located in tissues other than hemolymph. JH esterase, and to a lesser extent EH activity, has been characterized in a variety of insect species (Hammock, 1985; Roe and Venkatesh, 1990; Gilbert et al., 2000).

A majority of information about JH metabolism is the result of hemolymph JH esterase studies. The correlation of high levels of hemolymph JH esterase activity with low levels of JH during development, along with the disruption of normal development by JH esterase inhibitors suggests that JH titer, and therefore metamorphosis, is modulated by JH esterase (Roe and Venkatesh, 1990; Jones et al., 1990; Roe et al., 1997). Because of these findings, and the lack of research on JH metabolism in tissues other than hemolymph, JH esterase was considered to be the primary path of JH degradation in most insects studied (Hammock, 1985). Despite this earlier conception, evidence has been mounting slowly to support a hypothesis that EH in some insects may be as important as JH esterase in JH metabolism. For instance, a whole body study in *Musca domestica* (house fly), demonstrated that developmental profiles of EH and JH esterase activities were correlated (Yu and Terriere, 1978a). More recently, similar correlations with both

JH-metabolizing enzymes were found in some tissues of *Manduca sexta* (tobacco hornworm; Jesudason et al., 1992) and *Trichoplusia ni* (Wing et al., 1981; Kallapur et al., 1996). Furthermore, Kallapur et al. (1996) discovered that peak levels of EH were nearly equal to peak levels of JH esterase activity *in vitro* against JH III in *T. ni* fat body, midgut and integument throughout the fifth stadium. Also in *T. ni*, Hanzlik and Hammock (1988) showed that whole body EH and JH esterase activity was roughly equivalent in third and fourth stadium larvae. Perhaps the most convincing evidence for a primary role of EH in *in vivo* JH metabolism was presented by Halarnkar et al. (1993). This research determined that a phosphate conjugate of JH diol, not JH acid-diol, was the principal end product after *M. sexta* were injected with JH I. Unlike JH esterase, however, the determination of a direct role for EH in metamorphosis via JH degradation has remained elusive, mainly due to the lack of selective *in vivo* EH inhibitors (de Kort and Granger, 1996). Until such inhibitors of EH are available, other methods of research are necessary to provide clues on the importance of EH in comparison to JH esterase in JH metabolism.

Little or no information is available on EH regulation in insects. On the contrary, JH esterase regulation has been studied in several insect species (Hammock, 1985; Venkatesh and Roe, 1989; Roe and Venkatesh, 1990; Browder et al., 2001). Among those studies are reports on the effects of starvation on JH esterase activity and development, as well as indications from *in vivo* induction experiments that JH esterase is regulated by JH. In the current study, we examined developmental regulation during the whole life cycle, possible adult sexual dimorphism, and the effects of starvation and the exogenous application of insect hormones and xenobiotics on EH activity in the cabbage looper, *T. ni*.

MATERIALS AND METHODS

Insects

Cabbage loopers, *Trichoplusia ni* (Lepidoptera: Noctuidae), for this study originated from a USDA laboratory strain (Brownsville, Texas). Eggs were purchased from EntoPath, Inc. (Easton, PA). Larvae were reared on Southland *T. ni* artificial diet (Southland Products, Lake Village, AR) in 8 oz styrofoam cups. For general rearing and experiments, *T. ni* were held in an incubator at 27 $\pm$ 1^{o}C with relative humidity at 50 $\pm$ 5%, and a 14 h light:10 h dark cycle. Under these rearing conditions, the first (L1) through fourth (L4) larval stadia each were approximately 2 d (designated day 1 [D1] and day 2 [D2]) in duration. The last (fifth, L5) stadium lasted four days (D1 – D4), with the time immediately following ecdysis designated as L5D0. Neonate larvae marked the beginning of L1, and the age of L2-L5 larvae were determined by the occurrent times of head capsule slippage and ecdysis between each stadia. Head capsule slippage (appearance of a "bubblehead") began late on day 2 of L1-L4 stadia, at approximately 8-12 h before ecdysis. For experiments with L5 *T. ni*, L4 bubblehead larvae were held separately without food until ecdysis to provoke the majority of L5 test insects to be gate 1 (according to Kallapur et al., 1996). Following ecdysis, L5D0 larvae were provided with diet. L5D3 larvae were easily identified by their wandering behavior, cessation of feeding and migration to the side or top of the rearing container. Larvae then became prepupae on L5D4 and underwent ecdysis to pupae by the next day. For colony propagation, pupae and adults of mixed sexes were held in one-gallon cardboard containers with transparent lids. When virgin adults were needed, pupae first were sexed

and then males and females were held in separate containers until the time of experiments. After eclosion, adults were provided with honey:water (50:50) *ad libitum*.

JH Metabolism During Development

Eggs were collected for assay at 40-48 h after oviposition. Larvae assayed for JH esterase and JH EH were L1D2, L2D2, L3D2, L4D2, L5D0, L5D1, L5D2, L5D3 and L5D4. Pupae were sexed at 48-56 h after the larval-pupal ecdysis. Additional male and female pupae were allowed to emerge as adults. At 48-56 h after eclosion, wings from adults were excised. Insects were weighed and then stored at –80^{o}C until assayed for EH and JH esterase activity.

Starvation Experiments

Newly molted, unfed L5D0 larvae were weighed and individually placed in wells (3.0 ml volume) of 6-well plastic culture plates (Fisher Scientific; Fairlawn, NJ). One group was provided with the nutritive Southland *T. ni* diet. Another group was provided with non-nutritive diet consisting of 2.14% (w/v) agar (Moorhead & Co., Van Nuys, CA) and 0.71% (w/v) alphacel (non-nutritive cellulose bulk; ICN Biomedicals, Inc., Aurora, OH) in water. Immediately after the provision of diet, the 6-well plates were covered with plastic lids and placed in partially closed plastic bags to minimize dehydration of diet. All insects were allowed to consume diets *ad libitum*. Larvae from each treatment group were collected at 6, 28 and 52 h after the experiment began. Insects were weighed and then stored at –80^{o}C until assayed for EH and JH esterase activity.

Topical Treatments

Newly molted, unfed L5D0 larvae were weighed and treated topically with 1 μl ethanol or 50 μg in 1 μl ethanol of either JH III (75% purity; Sigma; St. Louis, MO), methoprene (98% purity; Chem Service; West Chester, PA), *trans*-stilbene oxide (TSO; 98% purity; Aldrich; Milwaukee, WI) or 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP; > 98% purity; synthesized in our laboratory according to Linderman et al., 1987). These solutions were applied to larvae three times a day. Insects were allowed to feed *ad libitum* in 6-well plastic culture plates maintained as described previously. The final topical applications were made at approximately 4 h prior to the end of the experiment. Larvae were collected at 28 and 52 h after the initiation of treatments. Insects were then weighed and stored at –80°C until assayed for EH and JH esterase activity.

Oral Treatments

Newly molted, unfed L5D0 larvae were weighed and individually placed in 6-well plastic culture plates and held as described previously. Larvae were provided with diet *ad libitum* containing 0.25% (w/w) methoprene (98% purity), 0.10% (w/w) TSO (98% purity), 0.10% (w/w) *cis*-stilbene oxide (CSO; 97% purity; Aldrich), 0.25% (w/w) sodium phenobarbital (98% purity; Gaines Chemical Co.; Pennsville, NJ), 0.25% (w/w) clofibrate (98% purity; Sigma) or 0.25% (w/w) cholesterol 5α,6α-epoxide (≥ 98% purity; Sigma). TSO and CSO were administered at 0.10% (w/w) due to total feeding repellency at 0.25% (w/w). To incorporate compounds in the food, molten diet was dispensed (0.3 ml per well) into 96-well plastic microtiter plates. After cooling, the plates were frozen at –80°C and lyophilized according to Bailey et al. (2001) for 48 h. The dehydrated diet

pellets were sealed in plastic bags and stored in a dessicator in the dark until needed for experiments. Except for phenobarbital, all compounds were dissolved in acetone and applied to the lyophilized diet pellets. The volume of acetone (150 μl) was sufficient to completely saturate the pellet without causing run-off. Pellets were held at room temperature under a chemical fume hood to allow for evaporation of acetone. The volume of fresh diet per well (300 μl) consistently lost 78-82% of the water content by weight during the lyophilization process. Therefore, pellets were rehydrated with 240 μl of distilled water or phenobarbital in distilled water for at least 1 h before the start of assays. For control and phenobarbital treatments, pellets were treated with acetone only and then rehydrated as described above. Larvae were collected at 28 and 52 h after the initiation of treatments. Insects were then weighed and stored at –80^{o}C until assayed for EH and JH esterase activity.

Preparation of Whole Body Homogenates

Frozen *T. ni* were homogenized in ice-cold sodium phosphate buffer (0.1 M, pH 7.4, 0.01% phenylthio urea [PTU]). For the starvation, oral and topical treatment experiments, larvae were homogenized with a Polytron PT10/35 homogenizer (Brinkmann Instruments, Westbury, NY) at the rate of 1.0 g body weight/1.5 ml buffer. Insects in the developmental study were homogenized with either a Kontes Duall (0.3 ml working capacity) or a Kontes Tenbroeck (7 ml working capacity) glass-on-glass tissue grinder (Kontes Glass Co., Vineland, NJ). These samples were homogenized at the rate of 1.0 g body weight/1.5 ml buffer, except for L1D2 larvae and adults, which were prepared at 1.0 g body weight/3.0 ml. All homogenates were centrifuged at 10,000 *g* for

20 min at 4°C, and the supernatants were then removed and filtered through glass wool (hexane-rinsed and dried). Clarified supernatants were aliquoted and stored at –80°C until assayed for enzyme activity.

Epoxide Hydrolase and JH Esterase Activity Assays

Clarified supernatants of homogenates were thawed on ice and diluted to enzyme concentrations that produced linear increases in enzymatic activity with time at 30°C. Each sample homogenate was assayed for EH and JH esterase by the partition assay of Share and Roe (1988). Homogenate (100 μl) was preincubated at 30°C for 10 min with either 1 μl of ethanol or 1 μl of 10 mM OTFP in ethanol. OTFP at a final assay concentration of 0.1 mM effectively inhibited 100% of the *T. ni* JH esterase activity without significantly affecting the rate of JH epoxide hydration. This was determined by the TLC method of Share and Roe (1988). Following preincubation with the inhibitor, the homogenates were incubated at 30°C with substrate consisting of [^{3}H]-racemic JH III (12 Ci/mmol, tritiated at C10; PerkinElmer Life Sciences, Boston, MA) and unlabeled racemic JH III (Sigma). The final assay concentration of JH substrate was 5 μM. The reaction then was quenched with methanol (300 μl) and isooctane (250 μl), and the aqueous and organic phases analyzed separately by liquid scintillation. The difference between total metabolism (ethanol-treated) and the EH activity (OTFP-treated) is the JH esterase activity. Protein concentrations of insect homogenates were determined by the Bio-Rad assay (Bio-Rad Laboratories, 1977), using bovine serum albumin (Fraction V; Fisher Scientific) as a standard. All samples were frozen and thawed only twice before assays. There was no loss of enzyme activity between insects homogenized and assayed

immediately, and that of samples frozen and thawed twice before enzyme analysis. Also, no significant differences were discovered in enzyme activity between fresh samples and those frozen up to 3 months.

RESULTS

EH vs. JH Esterase During T. ni Development

EH and JH esterase activity per mg protein was compared in whole body homogenates of eggs, all five larval stadia (L1-L5) and both sexes of pupae and virgin adults of *T. ni* (Fig. 2.1). For all data comparisons, significant differences were determined by t-tests ($\alpha = 0.05$). Both enzymes displayed measurable hydrolytic activity against JH III substrate at all life stages examined. JH esterase was significantly higher (1.5- to 3.3-fold) than EH activity at each time point except L5D3, when both enzymes were equal in activity. The highest levels of EH and JH esterase activity occurred at the egg stage. The EH activity for eggs was 2.10 nmol min^{-1} mg $protein^{-1}$, which was significantly (2.7-fold) higher than the second highest EH peak (0.78 nmol min^{-1} mg $protein^{-1}$) at L5D3. JH esterase activity in eggs was 3.32 nmol min^{-1} mg $protein^{-1}$. This peak was 2.0-fold higher and statistically different from the second highest level of JH esterase (1.65 nmol min^{-1} mg $protein^{-1}$) at L5D4. The lowest EH and JH esterase activities were present in adult males, at 0.09 and 0.19 nmol min^{-1} mg $protein^{-1}$, respectively. From the egg stage to L1D2, EH activity dropped 6.6-fold, while JH esterase decreased 5.6-fold. During the larval stages, EH activity ranged from 0.22 (L2D2) to 0.78 (L5D3) nmol min^{-1} mg $protein^{-1}$. With JH esterase, larval activity was lowest on L1D2 at 0.59, and highest on L5D4 at 1.65 nmol min^{-1} mg $protein^{-1}$. Following L5D4, EH and JH esterase activity decreased significantly for both sexes at the pupal stage. Female pupae had slightly higher EH activity than males, but JH esterase activities were not significantly different between the sexes. At adulthood, however, virgin females had 4.0- and 4.6-fold higher EH and JH esterase activities, respectively, than

virgin males. With females, EH and JH esterase activity was not different statistically between pupae and adults. Males, on the other hand, had 3.1- and 4.1-fold higher EH and JH esterase activity, respectively, in pupae when compared to adults.

Effects of Starvation on EH and JH Esterase

The effects of starvation on whole body EH and JH esterase activity and weight gain were evaluated over a 52 h period in *T. ni* larvae during the prewandering phase of the fifth stadium (Fig. 2.2). Beginning with newly molted, unfed L5D0 larvae, insects either were starved on a non-nutritive diet or fed standard *T. ni* artificial diet. When EH activity was measured per insect equivalent, larvae starved for 6 h had 48% of the activity of control insects fed the nutritive diet (Fig. 2.2A). By 28 and 52 h, EH activity in starved insects had only 5 and 1% of the control activity. Differences between treatments were not as dramatic when analyzed on a per mg protein basis. At 6 h after treatments began, EH activity between starved and control larvae was similar (Fig. 2.2B). At 28 and 52 h, however, EH activity in starved larvae was 46 and 25%, respectively, of the control.

With JH esterase, per insect equivalent (Fig. 2.2C) and per mg protein (Fig. 2.2D) activity of fed larvae was significantly higher than starved insects at all three time points after the initiation of treatments. JH esterase activity per insect equivalent was 29% of the control at 6 h, and then dropped substantially to 4 and 3% of the control at 28 and 52 h, respectively. Like EH activity, there were smaller differences between treatments when JH esterase activity was normalized per mg protein. In larvae starved for 6, 28 and 52 h, JH esterase per mg protein was 53, 40 and 71%, respectively, of the controls.

With all insects analyzed in enzyme assays, body weights were measured just before the beginning of treatments and again at the end of the experiment. The cumulative weight gain for each diet treatment at 6, 28 and 52 h is shown in Figure 2.2E. At these times, starved larvae gained only 37, 9 and 7%, respectively, of that gained by insects fed the nutritive diet. In fact, the weight of starved larvae only increased 18 mg in the first 6 h, and did not increase significantly from this point to the end (52 h) of the experiment.

Topical Induction of EH and JH Esterase

The effects of topically administered compounds on JH metabolism and weight gain in fifth stadium prewandering *T. ni* are displayed in Figure 2.3. L5D0 larvae were treated with 1 μl ethanol (control) or 1 μl compound three times per day over a 52 h period. Topically applied methoprene elicited the highest induction of EH activity per mg protein, with a 1.9-fold increase over control larvae at 28 h (Fig. 2.3A). TSO also was a significant inducer, 1.6-fold, at this time point. JH III and the JH esterase inhibitor, OTFP, both failed to significantly change EH activity. At 52 h, none of the compounds tested caused any change in EH activity as compared to control larvae.

Methoprene and JH III induced JH esterase activity per mg protein by 1.8- and 1.4-fold, respectively, at 28 h, and by 2.5- and 2.2-fold, respectively, at 52 h (Fig. 2.3B). Topically administered TSO had no impact on activity at either time point. OTFP inhibited JH esterase as expected, with 30 and 40% of the control activity detected at 28 and 52 h, respectively. When EH and JH esterase were analyzed on a per insect basis, trends in activity for all treatments were the same as compared to the respective data normalized per mg protein (data not shown).

Topically treated larvae also were evaluated for cumulative weight gain during the course of the experiment (Fig. 2.3C). Body weights were taken at the beginning and end of treatments. At 28 h after treatments began, JH III did not alter weight gain. Larvae treated with methoprene and TSO each gained 80% of the weight gained by the control. Those treated with OTFP gained 70% of the control weight. At 52 h, however, both JH III and methoprene caused a 1.3-fold increase in body weight gain. At the same time point, the body weight gain of larvae treated with TSO and OTFP was the same as the control treatment.

Oral Induction of EH and JH Esterase

The effects of orally administered compounds on JH metabolism and weight gain in fifth stadium prewandering *T. ni* are shown in Figure 2.4. Compounds were incorporated into artificial diet and fed *ad libitum* to newly molted, unfed L5D0 larvae for up to 52 h. Several compounds induced EH specific activity when compared to the control (Fig. 2.4A). As with topical treatments, methoprene was the best EH inducer, producing 2.5-fold higher EH activity than controls 28 h after treatments were initiated. At the same time point, CSO induced activity 2.0-fold, while TSO and phenobarbital each caused a 1.9-fold increase in EH activity. Clofibrate and cholesterol 5α,6α-epoxide did not increase EH activity at either 28 or 52 h after treatments began. The only compound to induce EH at 52 h was TSO, which elicited a 1.7-fold higher EH level as compared to the control.

Methoprene also was the best JH esterase inducer, with 2.5- and 1.8-fold elevation in activity per mg protien at 28 and 52 h, respectively (Fig. 2.4B). Methoprene was the only compound that significantly induced JH esterase activity at 28 h of feeding.

While TSO caused inhibition (70% of control) of JH esterase at 28 h, it actually induced activity significantly, 1.8-fold, after 52 h. Cholesterol 5α,6α-epoxide inhibited activity, having only 60% and 80% of control activity at the 28 and 52 h time points, respectively. CSO, phenobarbital and clofibrate had no significant impact on JH esterase activity at either time point. When EH and JH esterase were analyzed on a per insect basis, trends in activity for all treatments were the same as compared to the respective data normalized per mg protein (data not shown).

Cumulative weight gain also was determined during oral treatments (Fig. 2.4C). Body weights were taken at the beginning and end of the experiment. Of all treatments, only TSO at 28 h had any effect, with just 60% of the weight gain of control larvae. By 52 h, however, weight gain for *T. ni* treated with TSO was not significantly different from the control group.

DISCUSSION

EH and JH Esterase Activity Profiles During T. ni Development

Measurements of JH esterase activity in both hemolymph and other tissues of numerous insect species have been published extensively (Hammock, 1985; Roe and Venkatesh, 1990). While there are a number of reports that document EH activity in insects (Hammock, 1985; Mullin, 1988; Roe and Venkatesh, 1990), fewer studies characterize EH metabolism of JH during development, especially in comparison to JH esterase levels. JH metabolism by both enzymes has been investigated during development of *Spodoptera eridania* (southern armyworm; Slade et al., 1976), *M. domestica* (Yu and Terriere, 1978a), *Galleria mellonella* (wax moth; Wiśniewski et al., 1986), *M. sexta* (Share et al., 1988; Jesudason et al., 1992), *Culex quinquefasciatus* (southern house mosquito; Lassiter et al., 1995) and *T. ni* (Hanzlik and Hammock, 1988; Kallapur et al., 1996). Of these accounts, only the reports on *M. domestica* (Yu and Terriere, 1978a) and *T. ni* (Hanzlik and Hammock, 1988) measured *in vitro* JH esterase and EH activity in whole body – the other reports focused on enzyme activities in individual tissues or eggs. While Hanzlik and Hammock (1988) evaluated only third and fourth stadium *T. ni*, the current study investigated whole body JH III metabolism *in vitro* by EH and JH esterase at every life stage during *T. ni* development (Fig. 2.1). The peak of activity for both enzymes was at the egg stage. The EH activity in 2 d-old eggs was between 2.7- and 23.3-fold higher, while JH esterase was 2.0- to 17.5-fold higher than all other life stages. Also noteworthy is that JH esterase was 1.6-fold higher than EH activity in *T. ni* eggs. The opposite was discovered in 2 d-old *M. sexta* eggs, as EH was approximately 2.5-fold higher than JH esterase activity towards JH III (Share et al.,

1988). Despite high EH levels, Touhara and Prestwich (1994) concluded with *in vitro* experiments that JH esterase is likely the primary route of JH metabolism in *M. sexta* eggs. These authors proposed that JH is protected from EH metabolism by JH binding proteins, and that EH may be an ultimate scavenger of JH by hydrating JH acid to JH acid-diol. The high JH EH and JH esterase activity in *T. ni* eggs relative to other life stages suggests a significant role for both enzymes in JH metabolism during this stage. Unlike *M. sexta*, however, there currently is no information available on whether EH in *T. ni* eggs is involved in primary or secondary metabolism of JH.

During the larval stages of *T. ni*, whole body EH activity peaked on L5D3, although it was not statistically different from L5D1 and L5D2. JH esterase peaked on L5D4. Hanzlik and Hammock (1988) also showed in whole body *T. ni* that the highest JH esterase peak during larval development was on L5D4, but they also reported another peak in early L5D2 larvae. When separate tissues of *T. ni* fifth stadium larvae were analyzed *in vitro* for JH III metabolism, Kallapur et al. (1996) found that EH activity also peaked on L5D3 in fat body and integument, but was highest in midgut on L5D2. JH esterase in that study peaked on L5D2 in all three tissues tested, with a second, smaller peak on L5D4 only in fat body. As mentioned previously, Kallapur et al. (1996) showed as well that the peaks in EH activity were not different significantly from the peaks of JH esterase activity in *T. ni* fat body and midgut, and was actually higher in integument. In whole body third and fourth stadium *T. ni*, Hammock and Hanzlik (1988) also found nearly equal levels of EH and JH esterase activity. While our whole body *T. ni* data showed the presence of EH and JH esterase activity at all larval stages, JH esterase was higher (1.5- to 3.3-fold) than EH activity at all stages except L5D3. Nonetheless, both

EH and JH esterase were significant components of *in vitro* JH III metabolism in whole body *T. ni* during all larval stages.

Perhaps the most surprising analysis of JH metabolism during *T. ni* development was at the adult stage. While there was little or no difference between male and female pupae for either EH or JH esterase, there were dramatic differences in both enzymes between male and female adults. When 2 d-old whole body virgin adults were analyzed, females had 4.0- and 4.6-fold higher EH and JH esterase activity, respectively, than males. Female adult EH and JH esterase activities were not significantly different from female pupae, whereas male adults had significantly lower (3.1- and 4.1-fold) EH and JH esterase activities (respectively) compared to male pupae. In fact, male adults had the lowest levels of both enzymes throughout development. These results suggest that JH metabolism may not be important in regulating reproduction in *T. ni* males. There is some evidence that the presence of JH is necessary for proper development and function of the male reproductive system, including the supportive tissues, accessory glands, spermatogenesis and mating behavior (Koeppe et al., 1985). If this is the case, the low levels of EH and JH esterase witnessed in male adults may be vital in maintaining sufficient JH titers for male reproductive processes. Apparently this is not the case in all Lepidoptera, as Cole et al. (2002) reported in *Cydia pomonella* (codling moth) that virgin male adults had levels of JH esterase that were similar or significantly greater, depending on age, than virgin females. In female adults of *T. ni*, a high rate of JH metabolism seems to play a functional role in reproduction, based on our results and those of Venkatesh et al. (1988). The latter account reported that hemolymph JH esterase activity was high in *T. ni* adult virgin females, but then declined following mating. Similar results were noticed in *Heliothis virescens* (tobacco budworm; Ramaswamy et al., 2000). It is

believed that high JH metabolizing enzymes in these female moths maintain a low JH titer, which in turn inhibits egg development and oviposition until after mating. Following mating, a decline in JH metabolizing enzymes, coupled with an increase in JH titer, is likely responsible for initiating vitellogenesis (Englemann, 1979; Koeppe et al., 1985) and egg development, and increasing ovipositional rate (Venkatesh et al., 1988; Roe and Venkatesh, 1990). While these events appear to occur in female *T. ni* and *H. virescens*, again there is evidence that the role of JH metabolism in reproduction may vary among Lepidoptera. The previously mentioned study with *C. pomonella* also determined that JH esterase levels in virgin and mated adult females were the same, and that temporal fluctuations in JH titer seemed to be independent of JH esterase activity (Cole et al., 2002). Although JH esterase appears to be involved in reproduction of *T. ni* females, research is scarce on the involvement of EH. Based on our results, the significantly higher EH and JH esterase activity in whole body virgin female adults compared to males may implicate both enzymes in regulating reproduction in females, but not males. In *T. ni* females, studies that compare EH activity between virgin and mated moths are needed to corroborate EH involvement.

Effects of Starvation on EH and JH Esterase

Documentation of the effects of starvation on EH activity in insects is lacking. In fact, reports are rare on the consequences of starvation on EH activity in other animals as well. One such account with rats has shown that starvation for 48 h or 5 d reduced liver microsomal EH activity to 57% and 61% (respectively) of the controls (Thomas et al., 1989). In our study, it was of interest to discover if a similar effect of starvation occurs with EH activity during the fifth stadium of *T. ni*, starting with L5D0. Such information

could shed light on how JH-metabolizing EH in insects is regulated, as compared to JH esterase. In larvae that were starved on a non-nutritive diet, levels of both EH and JH esterase were reduced significantly as compared to control insects fed a standard nutritive diet (Fig. 2.2). Differences between the two treatments for both enzymes were greater when measured on a per insect equivalent basis (Fig. 2.2A and 2.2C), as compared to data normalized per mg protein (Fig. 2.2B and 2.2D). After just 6 h of starvation, EH and JH esterase activities per insect were 48 and 29% of the respective control activities. Levels of both enzymes dropped to only 1-5% of controls after 28 and 52 h of starvation. On a per mg protein basis, 6 h of starvation caused JH esterase activity to drop to nearly half that of fed larvae. Conversely, EH specific activity was not statistically different between the two treatments at the same time point. After 28 h of starvation, both enzymes were reduced to similar levels, as EH was 46% and JH esterase was 40% of the control activity. When larvae were starved for 52 h, EH activity was 25% and JH esterase was 71% of the activity of fed controls. The larger differences between treatments in per insect activity compared to per mg protein activity are likely due to the fact that fed individuals had substantially more (3.6-fold at 48 h) biomass and higher (18.5-fold at 48 h) protein concentration than starved insects.

When JH esterase was analyzed in the hemolymph of L5D1 *T. ni*, Venkatesh and Roe (1989) discovered that 18 h of starvation decreased JH esterase activity per mg protein to only 3% of fed larvae. This reduction was more significant than the diminishment of whole body JH esterase activity per mg protein observed in our experiments. The whole body homogenates obviously included fat body, which is believed to be the source of hemolymph JH esterase (reviewed by Hammock, 1985). Based on the hemolymph study and our whole body data, the major effect of starvation

on *T. ni* JH esterase may be cessation of its release from the fat body into the hemolymph, rather than a severe reduction of overall levels in the whole body. Similar dynamic compartmental effects of starvation are not probable with EH, as this enzyme appears to be only membrane-bound in insects.

It is well known that starvation at critical life stages can delay development in some insect species. This ability to delay pupation, in Lepidoptera for example, allows the insect to increase the length of the feeding stage until sufficient dietary reserves are obtained to survive the pupal stage and reproduce (Hammock, 1985). In *G. mellonella* (Reddy et al., 1979), *M. sexta* (Cymborowski et al., 1982; Sparks et al., 1983; Venkatesh and Roe, 1988; Browder et al., 2001) and *T. ni* (Hammock et al., 1981; Venkatesh and Roe, 1989), it was observed that, concurrent with delayed development, starvation caused a decrease in JH esterase activity. Other studies have determined that starvation also increased JH titer in *M. sexta* (Cymborowski, 1982) and *T. ni* (Jones et al., 1990). It is believed that the elevated JH titer during starvation is a result of the depression in JH esterase activity, and an abnormally long period of JH synthesis and secretion. When JH titer is not reduced at critical larval stages, prothoracicotropic hormone (PTTH) secretion is blocked, and this in turn causes the delay in metamorphosis (Nijhout and Williams, 1974b; Cymborowski et al., 1982; Hammock, 1985; Browder et al., 2001). In the current study, significant reductions in EH and JH esterase activity following starvation implies a role for both enzymes in regulating JH titer, and in turn, development.

Effect of Starvation on Body Weight

As expected, there were extreme differences in body weights between starved and fed larvae (Fig. 2.2E). The body weight of fed insects increased approximately 500%

just 52 h after the beginning of the experiment, while that of starved larvae increased only 33% in the first 6 h, and then did not change significantly from that point onward. This small weight gain (18 mg) in starved larvae only at the beginning of the experiment indicated that this was not an increase in biomass. Rather, this gain is likely an artifact from the ingestion of non-nutritive diet ingredients (water, alphacel and agar). Consequently, starved insects never showed any morphological signs of development during the course of the experiment. Larvae that were starved on non-nutritive diet but not collected earlier for samples were dead by 60 h after treatment initiation.

Hormonal Induction of EH and JH Esterase

There is clear evidence that JH is a specific substrate for JH esterase (Gilbert et al., 2000). It also is believed that JH itself is involved in the regulation of JH esterase. The induction of JH esterase by exogenous JH in several insect species, along with experiments involving head ligation, allatectomy and JH replacement, has led to the deduction that JH esterase is regulated at least in part by JH (Hammock, 1985; Roe and Venkatesh, 1990). More recently, experiments at the genetic level also have suggested a role for JH regulation of JH esterase. JH analogs appear to increase JH esterase gene transcription in *T. ni* (Venkataraman et al., 1994) and gene expression in *H. virescens* (Wroblewski et al., 1990). In *T. ni*, previous studies demonstrating JH esterase induction by JH have focused on the hemolymph (Sparks and Hammock, 1979; Jones and Hammock, 1983), or an *in vitro* fat body culture (Jones et al., 1987). Results of our experiments revealed that topically applied JH also induced JH esterase activity in *T. ni* whole body, at 28 and 52 h after treatment initiation (Fig. 2.3B). Additionally, the JH analog, methoprene, induced whole body JH esterase activity at 28 and 52 h with topical

(Fig. 2.3B) or oral (Fig. 2.4B) routes of administration. Methoprene induction of JH esterase has been documented in several insect species, including *Leptinotarsa decemlineata* (Colorado potato beetle; Kramer, 1978), *Ostrinia nubilalis* (European corn borer; Bean et al., 1983), *M. sexta* (Venkatesh and Roe, 1988; Jesudason et al., 1992) and *C. quinquefasciatus* adults (Lassiter et al., 1996). As expected, larvae treated with methoprene displayed typical juvenoid characteristics such as a prolonged fifth stadium, and death before or during the process of pupation.

Even though JH readily is metabolized by insect EH *in vitro*, no information is available on whether or not insect EH is regulated by JH. In order to provide clues on EH regulation in insects, the effects of exogenous JH III or methoprene on *T. ni* EH activity against JH substrate were analyzed. Contrary to JH esterase, EH activity was not induced significantly by topically applied JH III in whole body *T. ni* (Fig. 2.3A) at 28 h or 52 h after treatment initiation. Apparently, JH acid also did not induce EH activity at these time points. This is assuming that higher than normal levels of this metabolite are generated by induced JH esterase metabolism from topically applied JH. Surprisingly, methoprene induced EH activity at 28 h with topical (Fig. 2.3A) or oral (Fig. 2.4A) routes of administration. This may be the first documented case of EH induction by methoprene in insects. Previous trials with *M. sexta* (Jesudason et al., 1992) and *C. quinquefasciatus* (Lassiter et al., 1995, 1996) concluded that no EH induction occurred with methoprene. It is not fully understood why EH activity in *T. ni* was induced by the JH analog, but not JH. Perhaps a continuous dose of JH, or a higher concentration than methoprene is necessary for EH induction. Another possibility is that the topically applied JH may penetrate the cuticle quickly to associate with highly specific hemolymph JH-binding proteins that do not recognize methoprene. It has been postulated that these JH-specific

binding proteins protect JH from metabolic sources other than JH esterase (de Kort and Granger, 1996). This could explain why JH esterase, but not EH, was induced. Alternatively, the lack of JH induction may indicate that EH is not JH-responsive in *T. ni*. This would insinuate that methoprene induction of EH is a xenobiotic detoxication response, even though this compound lacks an epoxide. Many compounds that are not epoxides, and apparently are not metabolized to epoxides, induce microsomal EH (Wixtrom and Hammock, 1985). Based on the results of the present study, whole body JH esterase is inducible by JH, and likely is regulated by this hormone. However, a definitive conclusion cannot be made at this time on whether JH plays a role in EH regulation in *T. ni*.

Xenobiotic Induction of EH and JH Esterase

To investigate the potential of xenobiotic induction of JH-metabolizing enzymes in *T. ni* larvae, our topical (Fig. 2.3) and oral (Fig. 2.4) treatment experiments collectively included TSO, CSO, phenobarbital, clofibrate and OTFP. Both EH and JH esterase activity was evaluated to demonstrate any specificity of induction by these compounds. Previous studies have demonstrated EH induction by one or more of these xenobiotics in mites (TSO, CSO, phenobarbital: Cohen et al., 1993), insects (phenobarbital: Yu and Terriere, 1978b; Hällström and Grafström, 1981; Cohen, 1982; Yu and Hsu, 1985; Fuchs et al., 1992) and mammals (TSO, phenobarbital, clofibrate: reviewed by Wixtrom and Hammock, 1985). While the work of Yu and Terriere (1978b) showed in *M. domestica* that phenobarbital induced EH activity towards JH substrate, all the other reports above used other substrates. Therefore it was not known if the induced EHs in these other studies were capable of metabolizing JH as well. In our experiments with JH III

substrate, TSO induced *T. ni* EH significantly by either route of administration. Orally, CSO and phenobarbital also induced EH activity. Clofibrate, on the other hand, had no significant effect on EH levels. Topical treatments of the JH esterase inhibitor, OTFP, also did not alter EH activity. In addition to these xenobiotics, cholesterol 5α,6α-epoxide was analyzed by oral administration. Cholesterol epoxides are generated in plants (Meyer et al., 1998) and likely are ingested by insects. Moreover, insects may be exposed to the potentially toxic epoxides of cholesterol during metabolism of ingested plant sterols to cholesterol, and ultimately to ecdysone. There is evidence in mammals for a separate class of EH that is specific for metabolism of cholesterol epoxides (Watabe et al., 1981; Wixtrom and Hammock, 1985). This cholesterol EH is unique to the well-known microsomal and soluble EHs that are involved in xenobiotic detoxication. Because insects probably are exposed to cholesterol epoxides, they also might have cholesterol-specific EH. Our results support this hypothesis, since the EH measured in our *in vitro* assays metabolized JH, was induced by some xenobiotic epoxides and non-epoxides, but was not induced by cholesterol 5α,6α-epoxide.

Because JH esterase is specific for JH, it was unexpected that any of the xenobiotics would induce activity. When orally administered, CSO, phenobarbital and clofibrate made no significant changes in JH esterase activity, but TSO was inhibitory at 28 h, and inductive at 52 h. Topically applied TSO, however, did not change JH esterase activity at either time point. Although cholesterol 5α,6α-epoxide did not influence EH activity, it inhibited JH esterase. The rationale behind the affects of TSO and cholesterol 5α,6α-epoxide on JH esterase is unclear. OTFP was included in topical treatments as a negative control. As expected, this compound inhibited the majority of JH esterase

activity at both time points. In general, JH esterase was not induced by xenobiotics, other than the anomaly with orally administered TSO. EH on the other hand, was induced by multiple xenobiotics, including compounds with and without epoxides. These results suggest that *T. ni* EH plays a major role in xenobiotic metabolism, while JH esterase is specific for metabolism of JH.

Effects of Hormone and Xenobiotic Treatments on Body Weight

The effects of the topical or oral treatments on cumulative body weight gain also were monitored. During topical applications, methoprene, TSO and OTFP caused a significant reduction in body weight gain at 28 h after treatments began. Despite the reduction in body weight at this time point, methoprene caused induction of both EH and JH esterase, while TSO induced EH but had no impact on JH esterase. At 52 h after treatments started, however, JH III and methoprene elicited significant increases in body weight gain. The weight increase for both compounds resulted in no change in EH activity, but JH esterase induction by JH and methoprene was higher at this time point than at 28 h. For these topical assays, the dose of compound per insect was the same whether larval weight gain was reduced or increased. In oral experiments, the dosage is determined by the amount of treated diet that is consumed by each larva. Furthermore, the dose can differ between treatments based on the palatability of the compounds. In oral experiments, the body weight gain for all treatments was directly proportional to the weight of diet consumed (data not shown). Despite the possibilities of dose fluctuations in oral treatments, TSO was the only treatment that caused any significant change in body weight gain, and therefore dose. At 28 h after treatment began, TSO reduced body weight gain to 60% of the control. By 52 h, TSO was not different significantly from the

control. Structurally, CSO is quite similar to TSO, but CSO did not affect body weight gain. Both TSO and CSO were incorporated into diet at a lower concentration (0.10% w/w) than the other compounds (0.25% w/w) due to their total repellency of larval feeding at the higher amount. Regardless of the lower doses, both TSO and CSO were biologically active by induction of EH, and by inhibition (28 h) or induction (52 h) of JH esterase activity.

Summary

Both EH and JH esterase were components of *in vitro* JH III metabolism during all life stages of *T. ni*. However, JH esterase was higher than EH activity at all ages assayed except L5D3. The highest level of both enzymes was at the egg stage, which suggests that a high rate of JH metabolism is important in egg development. With whole body virgin adults, the significantly higher EH and JH esterase activity discovered in females as compared to males may implicate both enzymes in regulating female, but not male reproduction. Delayed development and significant reductions in EH and JH esterase activity following starvation of *T. ni* larvae implies a role for both enzymes in regulating JH titer, and in turn, larval development. In induction studies, JH esterase was inducible by JH III and the JH analog, methoprene, which suggests that this enzyme is regulated by JH. Because EH was induced by the JH analog and not by JH, a definitive conclusion can not be made at this time on whether JH plays a role in EH regulation. In general, induction of JH esterase by xenobiotics was rare, while EH was induced by multiple xenobiotics, including compounds with and without epoxides. These results suggest that EH, but not JH esterase, plays a major role in xenobiotic metabolism in larvae of the cabbage looper, *T. ni*.

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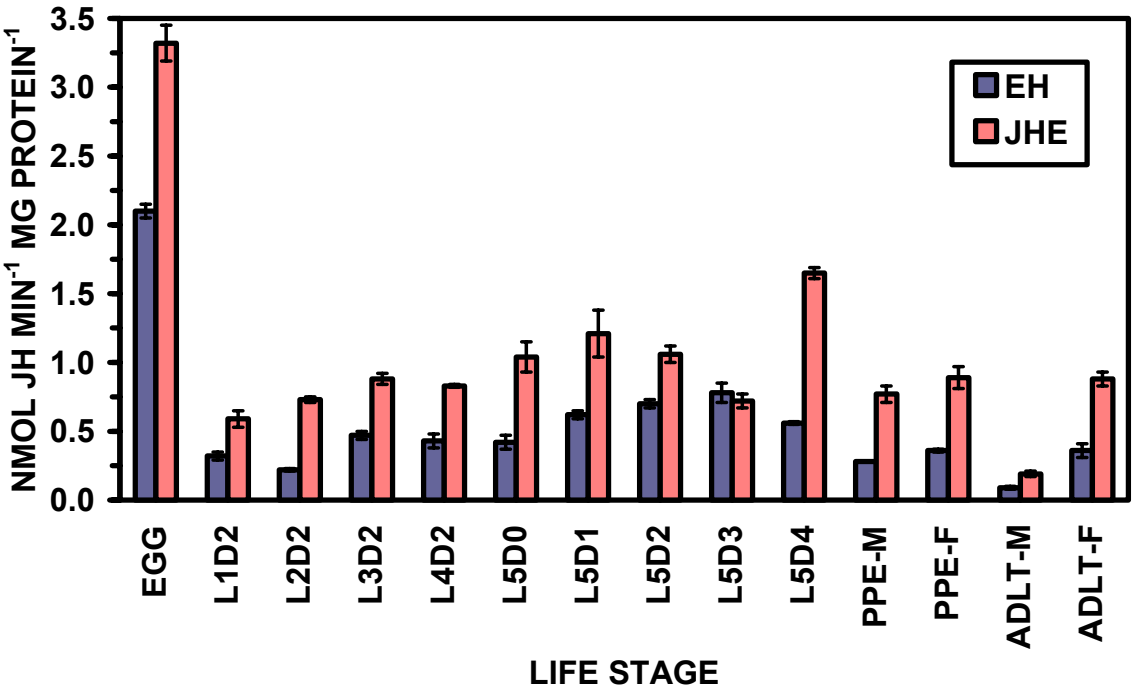


Figure 2.1. EH and JH esterase activity of whole body homogenates during *T. ni* development. [^{3}H]-JH III was used as the substrate. Insects were staged as described in Materials and Methods. Each datum point is the mean of 3 experiments that each consisted of 500-700 eggs or 5-30 larvae, pupae or adults. Error bars at $\pm$ 1 SEM are presented when larger than the datum point. L1 – L5 = first through fifth stadium, D0 – D4 = days 0 – 4 within a stadium; PPE-M = male pupae, PPE-F = female pupae; ADLT-M = male virgin adults, ADLT-F = female virgin adults.

Figure 2.2. Effects of starvation on JH metabolism in prewandering, fifth stadium *T. ni*. Initiation of dietary treatments (nutritive or non-nutritive diet) began with newly molted, unfed L5D0 larvae. Epoxide hydrolase and JH esterase activity was measured in whole body homogenates using [^{3}H]-JH III as the substrate. (A) EH activity per insect equivalent; (B) EH activity per mg protein; (C) JH esterase activity per insect equivalent; (D) JH esterase activity per mg protein; and (E) cumulative body weight gain from the beginning of treatments. Each datum point is the mean of 3 experiments that each consisted of 5 insects. Error bars for 1 $\pm$ SEM are present when larger than each datum point. For non-nutritive treatment data within each graph, the percent of control (nutritive diet) value is shown when the datum point is significantly different (t-test, α = 0.05) from the control at each time point.

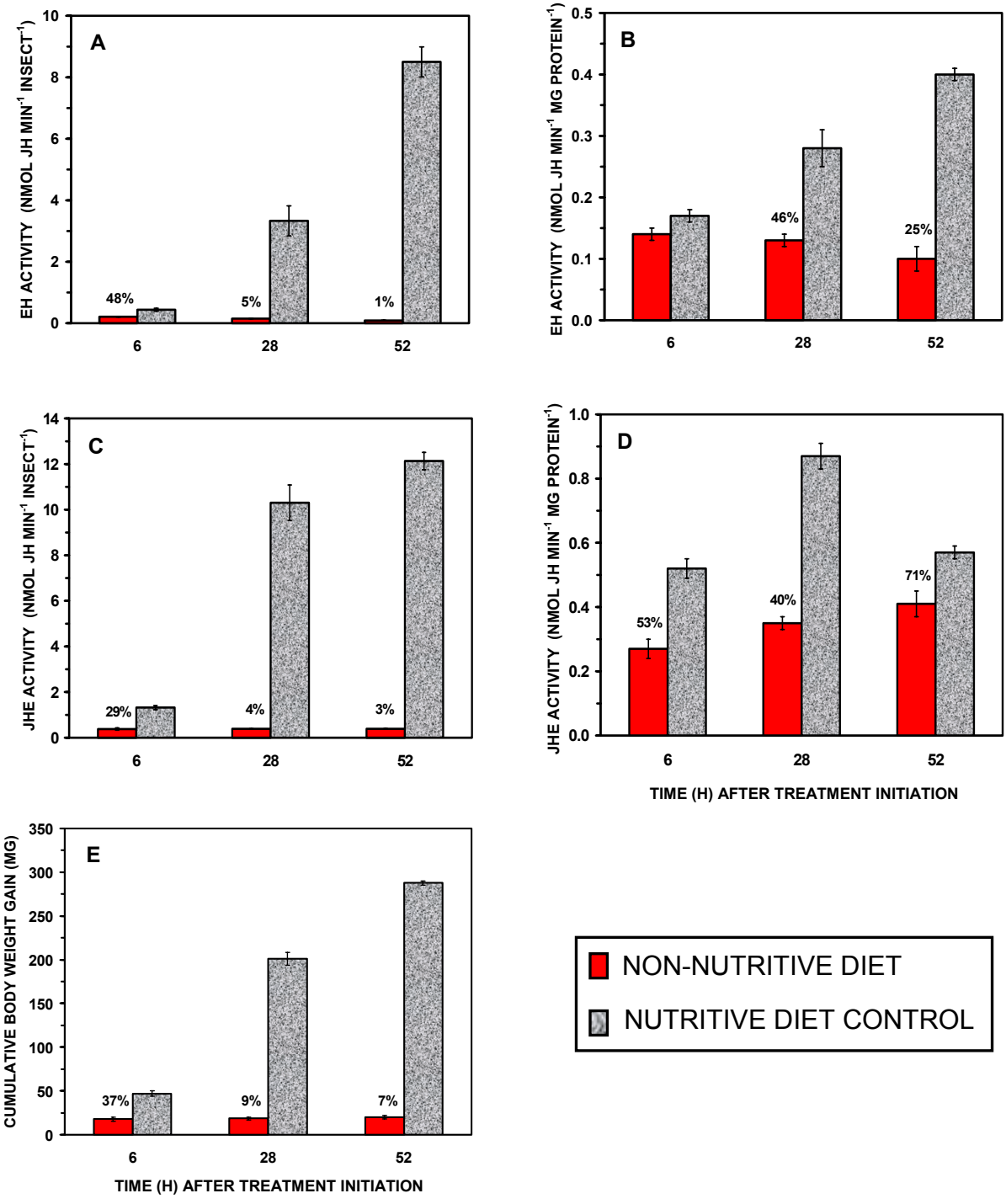

A
EH ACTIVITY (NMOL JH MIN-1 INSECT-1)
48%
5%
1%
B
EH ACTIVITY (NMOL JH MIN-1 MG PROTEIN-1)
46%
25%
C
JHE ACTIVITY (NMOL JH MIN-1 INSECT-1)
29%
4%
3%
D
JHE ACTIVITY (NMOL JH MIN-1 MG PROTEIN-1)
53%
40%
71%
6
28
52
TIME (H) AFTER TREATMENT INITIATION
E
CUMULATIVE BODY WEIGHT GAIN (MG)
37%
9%
7%
TIME (H) AFTER TREATMENT INITIATION
NON-NUTRITIVE DIET
NUTRITIVE DIET CONTROL

Figure 2.3. Effects of topically applied hormones and xenobiotics on JH metabolism in prewandering fifth stadium *T. ni*. One microliter of ethanol (control), or 1 μl of ethanol containing 50 μg of compound was applied 3 times per day beginning with newly molted, unfed L5D0 larvae. EH and JH esterase activity was measured in whole body homogenates using [^{3}H]-JH III as the substrate. (A) EH activity per mg protein; (B) JH esterase activity per mg protein; and (C) cumulative body weight gain from the beginning of treatments. Each datum point is the mean of 3-5 experiments that each consisted of 5 insects. Error bars are 1 $\pm$ SEM. For each topical treatment within each graph, the fold increase or percent of control value is shown when the datum point is significantly different (t-test, $\alpha = 0.05$) from the control at each time point. (TSO, *trans*-stilbene oxide; OTFP, 3-octylthio-1,1,1-trifluoropropan-2-one).

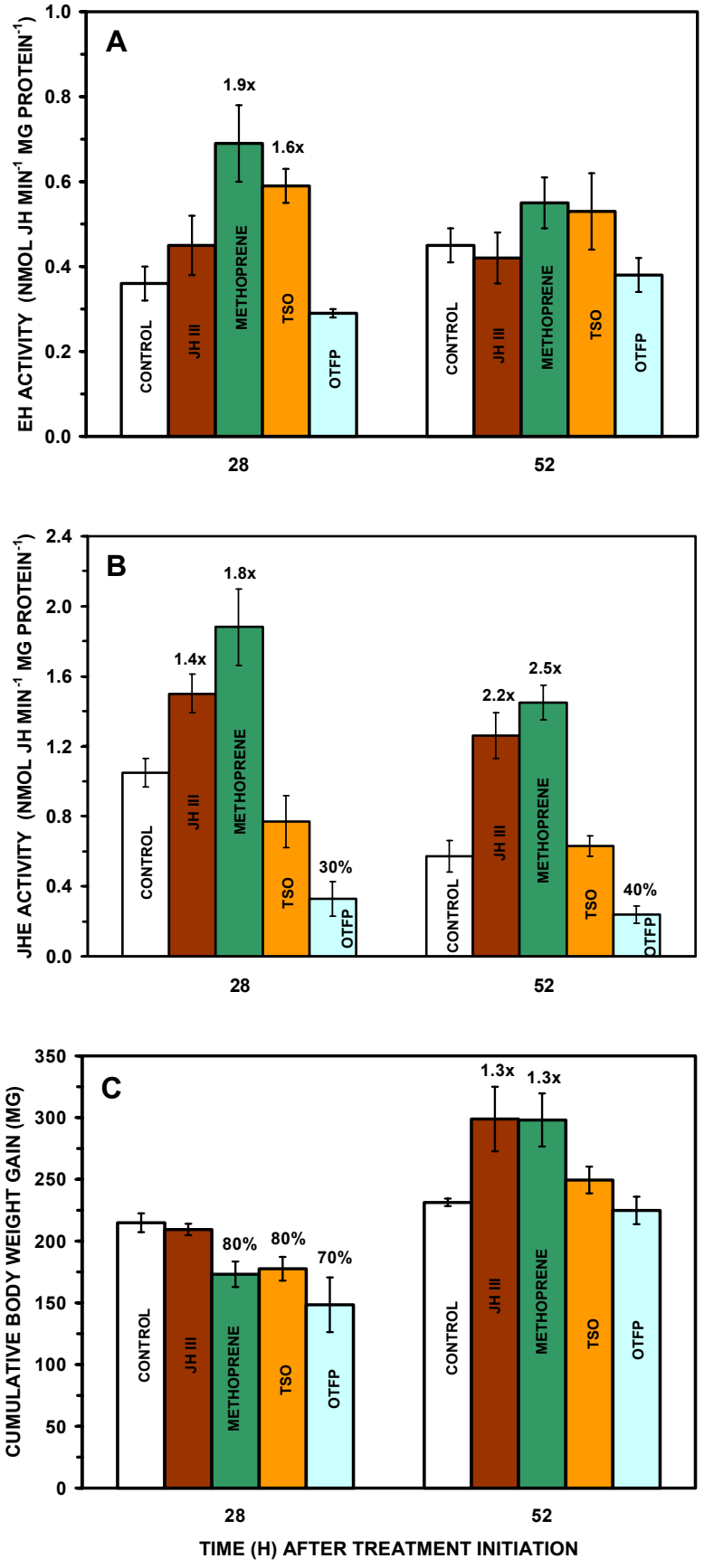
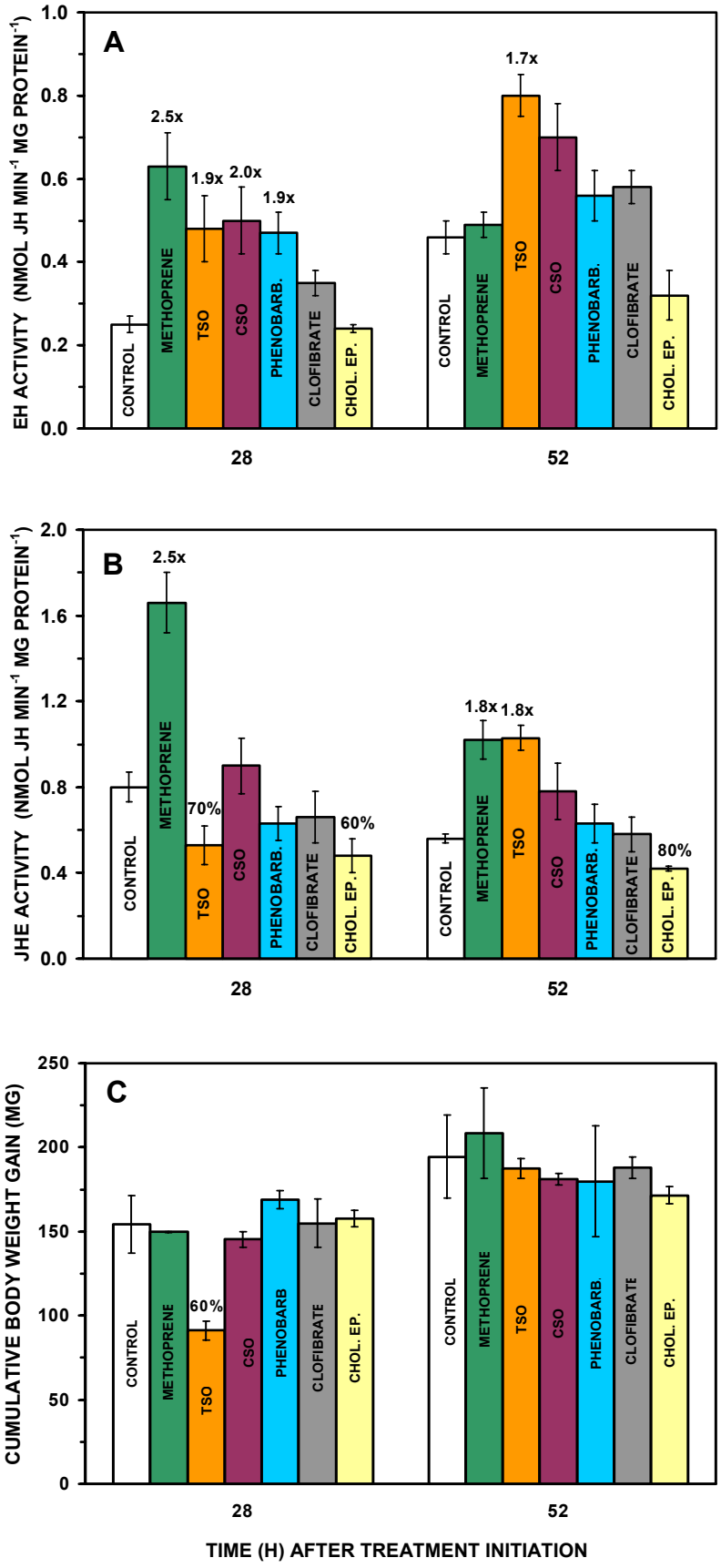

A
EH ACTIVITY (NMOL JH MIN-1 MG PROTEIN-1)
1.0
0.8
0.6
0.4
0.2
0.0
1.9x
1.6x
CONTROL
JH III
METHOPRENE
TSO
OTFP
28
52
B
JHE ACTIVITY (NMOL JH MIN-1 MG PROTEIN-1)
2.4
2.0
1.6
1.2
0.8
0.4
0.0
1.8x
1.4x
2.5x
2.2x
30%
40%
C
CUMULATIVE BODY WEIGHT GAIN (MG)
350
300
250
200
150
100
50
0
1.3x
1.3x
80%
80%
70%
TIME (H) AFTER TREATMENT INITIATION

Figure 2.4. Effects of orally administered hormones and xenobiotics on JH metabolism in prewandering fifth stadium *T. ni*. Compounds to be tested were incorporated in diet as described in the Materials and Methods. The diet concentration of methoprene, phenobarbital (phenobarb.), clofibrate and cholesterol 5α,6α-epoxide (chol. ep.) was 0.25% (w/w), while *trans*-stilbene oxide (TSO) and *cis*-stilbene oxide (CSO) were formulated at 0.10% (w/w). Larvae were fed *ad libitum* beginning at L5D0. EH and JH esterase activity was measured in whole body homogenates using [^{3}H]-JH III as the substrate. (A) EH activity per mg protein; (B) JH esterase activity per mg protein; and (C) cumulative body weight gain from the beginning of treatments. Each datum point is the mean of 3 experiments that each consisted of 5 insects. Error bars are 1 $\pm$ SEM. For each treatment within each graph, the fold increase or percent of control value is shown when the datum point is significantly different (t-test, $\alpha = 0.05$) from the control at each time point.


A
EH ACTIVITY (NMOL JH MIN-1 MG PROTEIN-1)
1.0
0.8
0.6
0.4
0.2
0.0
2.5x
1.9x
2.0x
1.9x
1.7x
CONTROL
METHOPRENE
TSO
CSO
PHENOBARB.
CLOFIBRATE
CHOL. EP.
28
52
B
JHE ACTIVITY (NMOL JH MIN-1 MG PROTEIN-1)
2.0
1.6
1.2
0.8
0.4
0.0
2.5x
70%
60%
1.8x
1.8x
80%
C
CUMULATIVE BODY WEIGHT GAIN (MG)
250
200
150
100
50
0
60%
CONTROL
METHOPRENE
TSO
CSO
PHENOBARB
CLOFIBRATE
CHOL. EP.
28
52
TIME (H) AFTER TREATMENT INITIATION

APPENDIX A

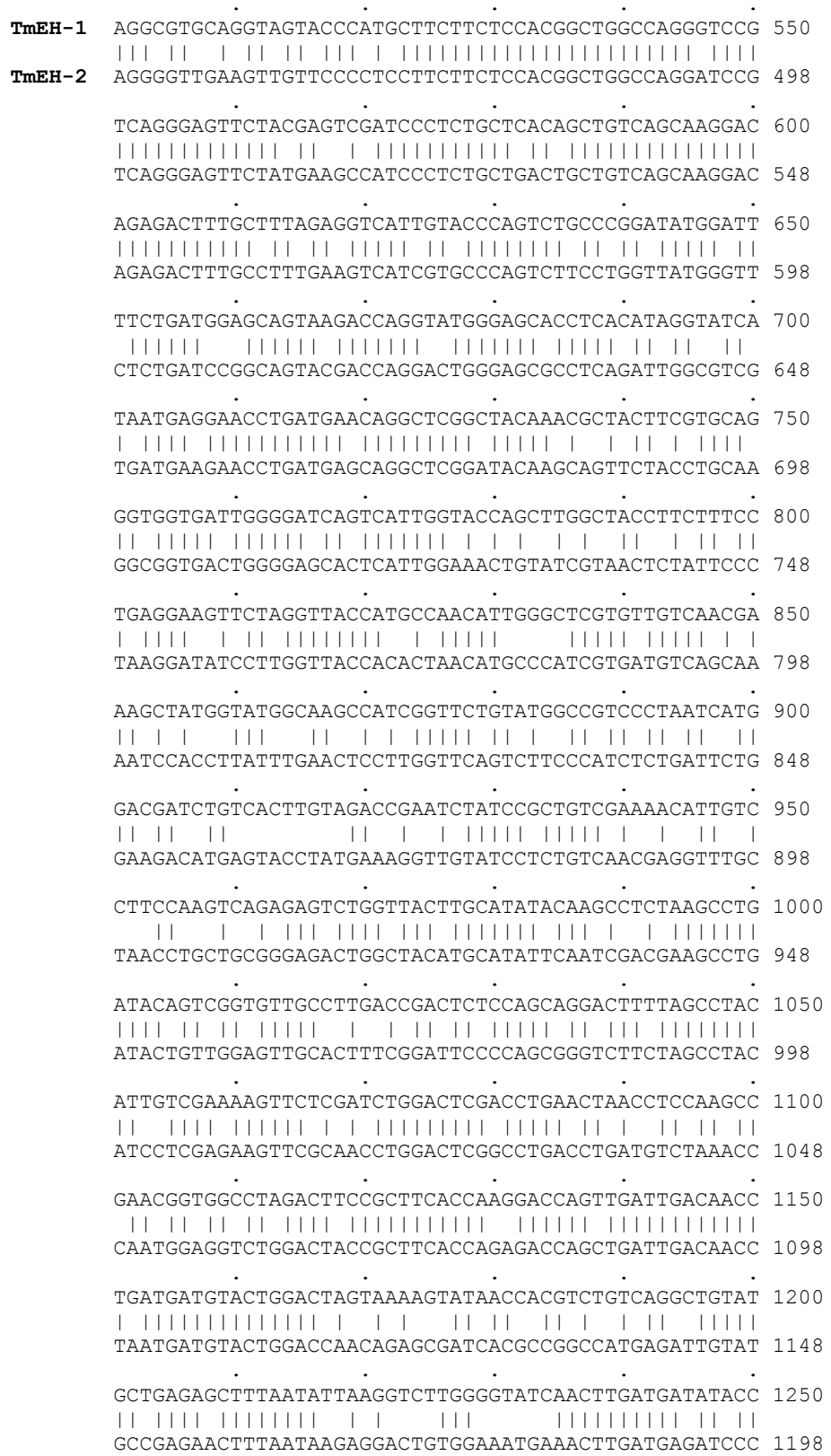
GAP AND CLUSTAL X SEQUENCE ALIGNMENTS

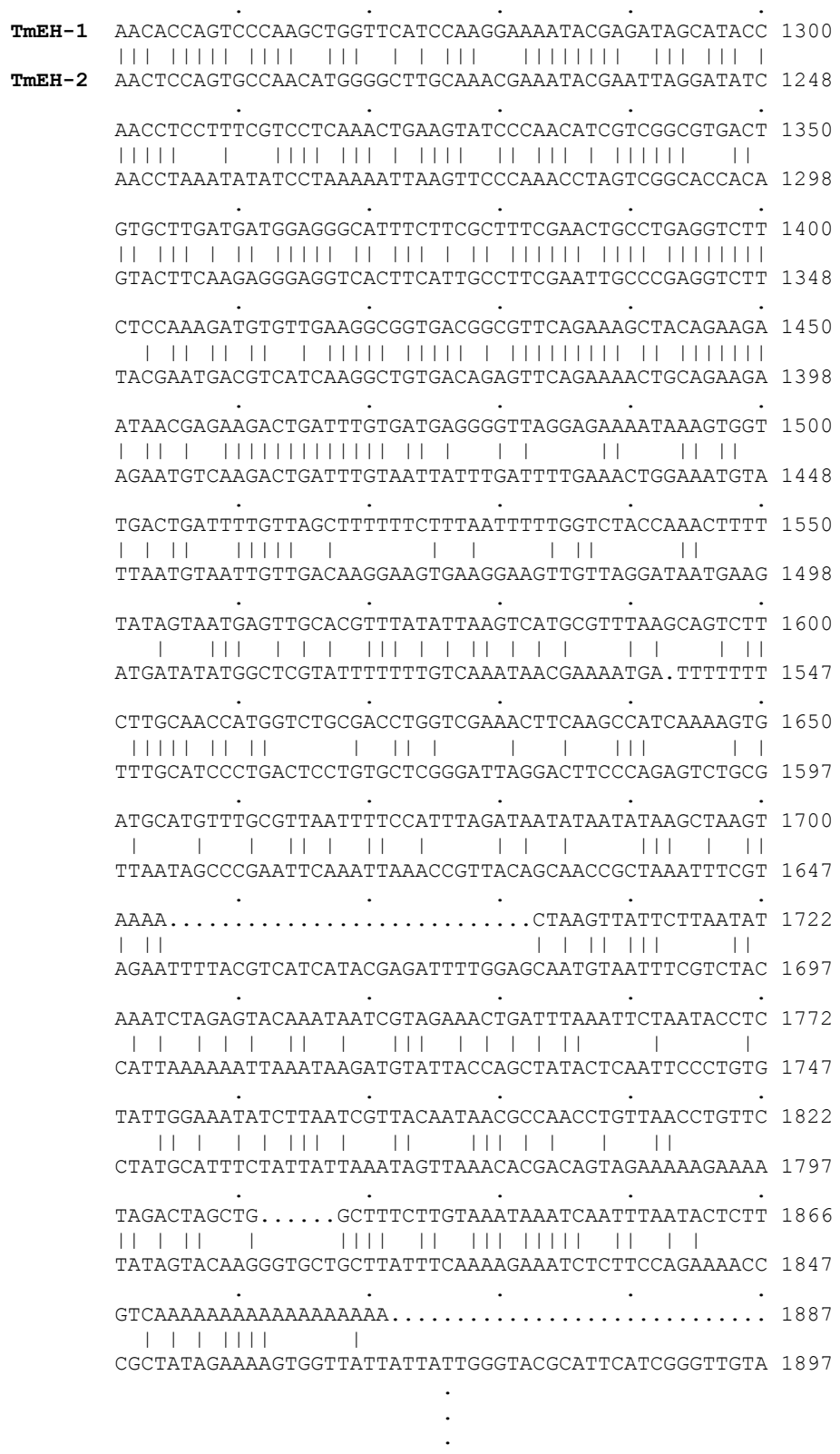
Comparisons of the Full-Length *Trichoplusia ni* Epoxide Hydrolase cDNA Isolated from Gut (TmEH-2) with a Selection of Other Known Epoxide Hydrolase Sequences

GAP Alignment (SEQWeb, version 1.1; GCG) of Nucleic Acid Sequences of the Two *Trichoplusia ni* Epoxide Hydrolases: TmEH-1 (Isolated from Fat Body; NCBI Accession #U73680) and TmEH-2 (Isolated from Gut; NCBI Accession #AF035482)

```
GAP of: TmEH-1 (from: 1  to: 1887) to: TmEH-2 (from: 1  to: 2054)

Symbol comparison table: /local/gcg10/gcgcore/data/rundata/nwsgapdna.cmp
CompCheck: 8760

        Gap Weight:     50      Average Match: 10.000
     Length Weight:      3   Average Mismatch:  0.000

           Quality:  12027             Length:   2107
             Ratio:  6.374               Gaps:      4
Percent Similarity: 67.339   Percent Identity: 67.339

       Match display thresholds for the alignment(s):
                   | = IDENTITY
                   : =   5
                   . =   1
```

```
                .         .         .         .         .
TmEH-1  TCAGATAGCGGTCGTGTGACTACGTAACACATTATTTATACATATTTAAC 50
                                                      |||
TmEH-2  ..........................................CAAC 4
                .         .         .         .         .
        TTTTGATAAAACGATTGTGAAGTTTTTTAATATGGGTCGCCTCTTATTCC 100
        |||| ||   |    ||      ||||||||||||  || ||| | |||
        TTTTTATTTCAATTGTG......TTTTTAATATGGCCCGTCTCCTCTTCA 48
                .         .         .         .         .
        TAGTGCCAGTATTGGCGATAGTCCTTCTGCCAGTATACTACCTATTCCTA 150
        || | ||||||||||||  | ||| |||| |||||||||| | ||||||||
        TACTACCAGTATTGGCACTGGTCTTTCTCCCAGTATACTTCTTATTCCTA 98
                .         .         .         .         .
        CAAGGTCCTCCACCATTACCAGACTTGGACTACAACGAGTGGTGGGGGCC 200
        ||| ||||||||||  ||||  |  | |||   ||||| ||||||||||
        CAAAGTCCTCCACCGGTACCCAATGTTGACATGAACGATTGGTGGGGGCC 148
                .         .         .         .         .
        TGAGAGCGGAAAACAAAAACAGGACACCAGCGTCAGGCCATTCAAAATCA 250
        ||||||||  ||| |||||||||| |||||  ||||||||||||||||||
        TGAGAGCGCGAAAGAAAAACAGGATACCAGTATCAGGCCATTCAAAATCA 198
                .         .         .         .         .
        ATTTTGGAGAAAATCTCGTTAAAGATTTGAAAGATCGCCTCAAGAGGACA 300
         ||||||| | |||  ||| ||||| || |||||||| ||| | || |||
        GTTTTGGAAATAATAACGTCAAAGACTTAAAAGATCGTCTCCAAAGAACA 248
                .         .         .         .         .
        AGACCTTTAACACCTCCATTAGAAGGAGTTGGCTTCGAATATGGCTTCAA 350
        |||||  |||||||||||| ||||||| || |||||||| || ||||||||
        AGACCGCTAACACCTCCACTAGAAGGTGTCGGCTTCGACTACGGCTTCAA 298
                .         .         .         .         .
        CACCAACGAAATTAATAGCTGGTTGAAGTACTGGGCTGAAGGATACAATT 400
        ||||||||||||  | ||||||||||||||||||||  | |  |||||||
        CACCAACGAAATCGACAGCTGGTTGAAGTACTGGGCCAAGGACTACAATT 348
                .         .         .         .         .
        TCAAGGAGAGGGAAACCTTCCTGAACCAGTTCCCGCAGTTCAAGACTAAC 450
        |||||||||||||||| ||| ||||||||||||||||||||||||||||
        TCAAGGAGAGGGAAACTTTCTTGAACCAGTTCCCGCAGTTCAAGACTAAC 398
                .         .         .         .         .
        ATCCAGGGTCTGGATATACACTTCATCAAAGTTACTCCTAAGGTCCCAGC 500
        |||||||| |||||||||||||||| |  ||||| || ||||| ||
        ATCCAGGGACTGGATATACACTTCATTAGGGTTACGCCAAAGGTTCCTCA 448
```


TmEH-1
TmEH-2

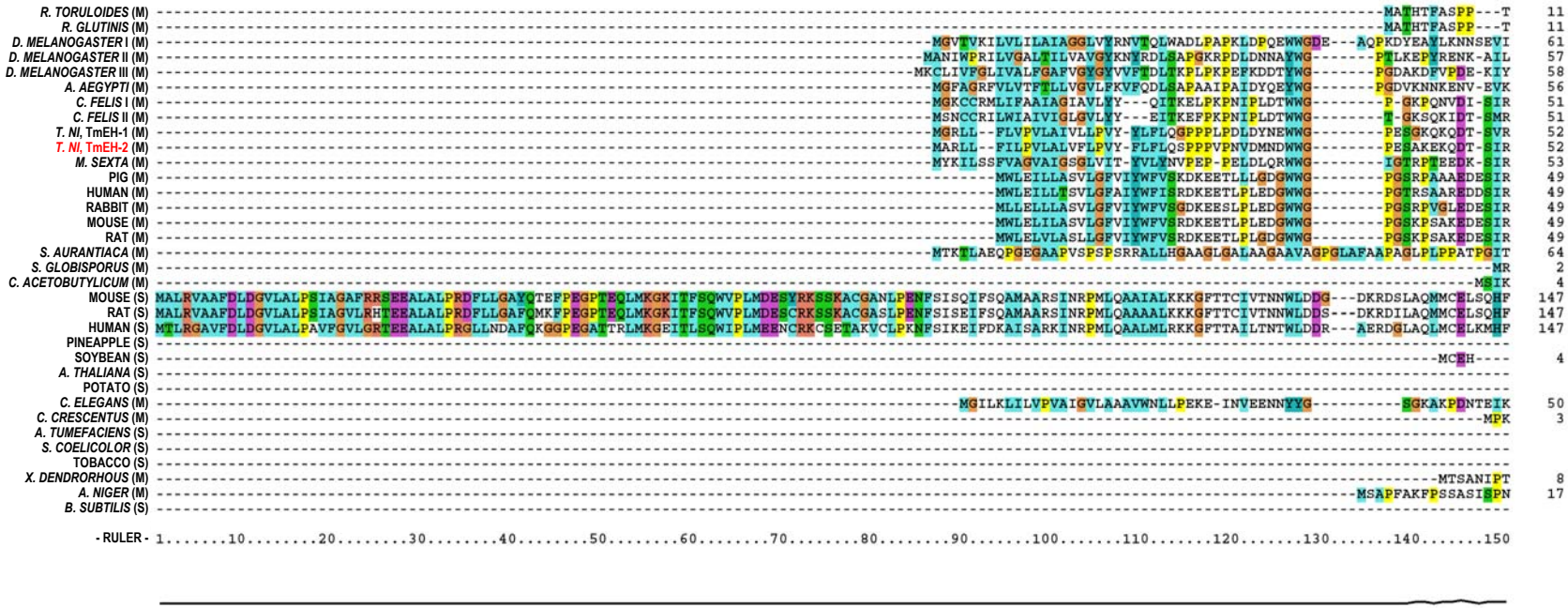

TmEH-1
TmEH-2

GAP Alignment (SEQWeb, version 1.1; GCG) of Deduced Amino Acid Sequences of the Two *Trichoplusia ni* Epoxide Hydrolases: TmEH-1 (Isolated from Fat Body; NCBI Accession #U73680) and TmEH-2 (Isolated from Gut; NCBI Accession #AF035482)

```
GAP of: TmEH-1 (from 1 to 463) to: TmEH-2 (from 1 to 463)

BLOSUM62 amino acid substitution matrix.
Reference: Henikoff, S. and Henikoff, J. G. (1992). Amino acid
           substitution matrices from protein blocks.  Proc. Natl. Acad.
           Sci. USA 89: 10915-10919.

         Gap Weight:      8      Average Match:  2.912
      Length Weight:      2   Average Mismatch: -2.003

            Quality:   1922            Length:    463
              Ratio:  4.151              Gaps:      0
 Percent Similarity: 82.073   Percent Identity: 72.570

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =   2
                    . =   1

                .         .         .         .         .
TmEH-1  MGRLLFLVPVLAIVLLPVYYLFLQGPPPLPDLDYNEWWGPESGKQKQDTS 50
        | ||||:.||||:| ||||:|||| |||.|..| |:|||||| |:|||||
TmEH-2  MARLLFILPVLALVFLPVYFLFLQSPPPVPNVDMNDWWGPESAKEKQDTS 50
                .         .         .         .         .
        VRPFKINFGENLVKDLKDRLKRTRPLTPPLEGVGFEYGFNTNEINSWLKY 100
        :|||||.|| | ||||||||.|||||||||||||:||||||||.|||||
        IRPFKISFGNNNVKDLKDRLQRTRPLTPPLEGVGFDYGFNTNEIDSWLKY 100
                .         .         .         .         .
        WAEGYNFKERETFLNQFPQFKTNIQGLDIHFIKVTPKVPAGVQVVPMLLL 150
        ||. |||||||||||||||||||||||||:|||||| ||:|||:|||
        WAKDYNFKERETFLNQFPQFKTNIQGLDIHFIRVTPKVPQGVEVVPLLLL 150
                .         .         .         .         .
        HGWPGSVREFYESIPLLTAVSKDRDFALEVIVPSLPGYGFSDGAVRPGMG 200
        ||||||||||||.|||||||||||||| |||||||||||||| |||||:|
        HGWPGSVREFYEAIPLLTAVSKDRDFAFEVIVPSLPGYGFSDPAVRPGLG 200
                .         .         .         .         .
        APHIGIIMRNLMNRLGYKRYFVQGGDWGSVIGTSLATFFPEEVLGYHANI 250
        || ||::|:|||.|||||.::.||||||..||  : | ||.::|||| |.
        APQIGVVMKNLMSRLGYKQFYLQGGDWGALIGNCIVTLFPKDILGYHTNM 250
                .         .         .         .         .
        GLVLSTKAMVWQAIGSVWPSLIMDDLSLVDRIYPLSKTLSFQVRESGYLH 300
         :|:| |. ..: :|||.||||::|:|  :|:||||   .  .||.||:|
        PIVMSAKSTLFELLGSVFPSLILEDMSTYERLYPLSTRFANLLRETGYMH 300
                .         .         .         .         .
        IQASKPDTVGVALTDSPAGLLAYIVEKFSIWTRPELTSKPNGGLDFRFTK 350
        ||..|||||||||.||||||||||.|||. ||||:| ||||||||:|||:
        IQSTKPDTVGVALSDSPAGLLAYILEKFATWTRPDLMSKPNGGLDYRFTR 350
                .         .         .         .         .
        DQLIDNLMMYWTSKSITTSVRLYAESFNIKVLGYQLDDIPTPVPSWFIQG 400
        ||||||||||||.:.|| ..|||||.|| : .  .||:||||||.| :|
        DQLIDNLMMYWTNRAITPAMRLYAENFNKRTVEMKLDEIPTPVPTWGLQT 400
                .         .         .         .         .
        KYEIAYQPPFVLKLKYPNIVGVTVLDDGGHFFAFELPEVFSKDVLKAVTA 450
        |||: ||| ::||:|:||:|| ||| :|||| ||||||||. ||:||||
        KYELGYQPKYILKIKFPNLVGTTVLQEGGHFIAFELPEVFTNDVIKAVTE 450
                .
        FRKLQKNNEKTDL 463
        |||||| | ||||
        FRKLQKKNVKTDL 463
```

CLUSTAL X (v. 1.64b) amino acid sequence alignment used to construct the phylogenetic tree (Fig. 1.2) of microsomal (M) and soluble (S) epoxide hydrolases from a selection of organisms* spanning four kingdoms

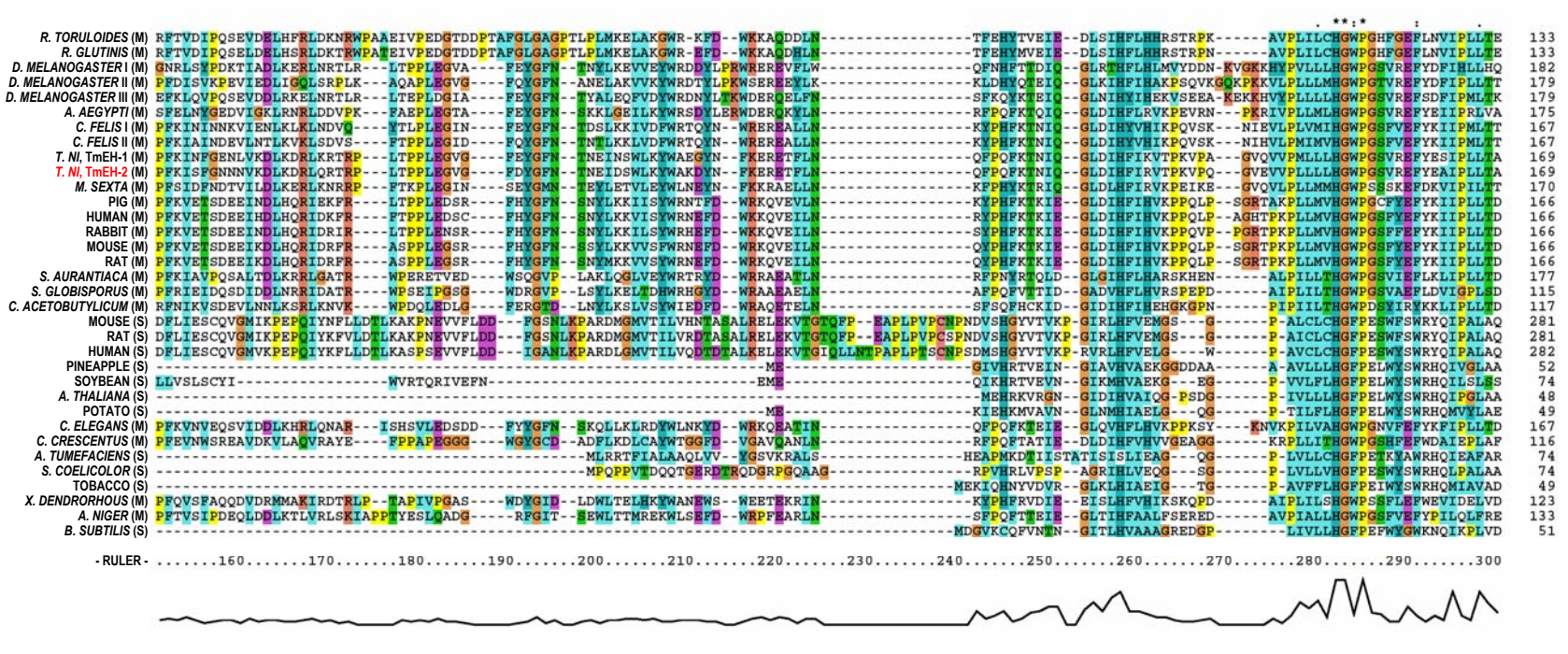
(Page 1 of 5)



* Complete scientific names and NCBI accession numbers of each organism are provided in Table 1.1.

CLUSTAL X (v. 1.64b) amino acid sequence alignment used to construct the phylogenetic tree (Fig. 1.2) of microsomal (M) and soluble (S) epoxide hydrolases from a selection of organisms* spanning four kingdoms

(Page 2 of 5)



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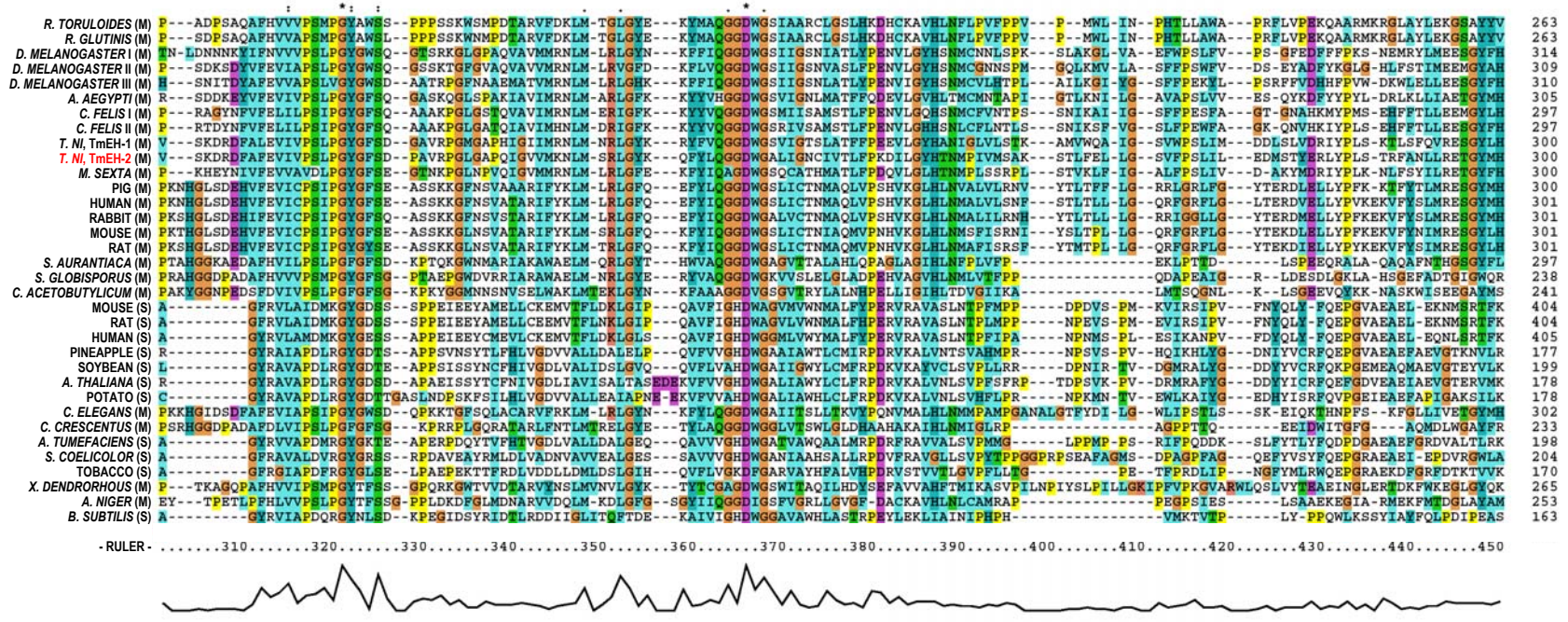
CLUSTAL X (v. 1.64b) amino acid sequence alignment used to construct the phylogenetic tree (Fig. 1.2) of microsomal (M) and soluble (S) epoxide hydrolases from a selection of organisms* spanning four kingdoms

(Page 3 of 5)



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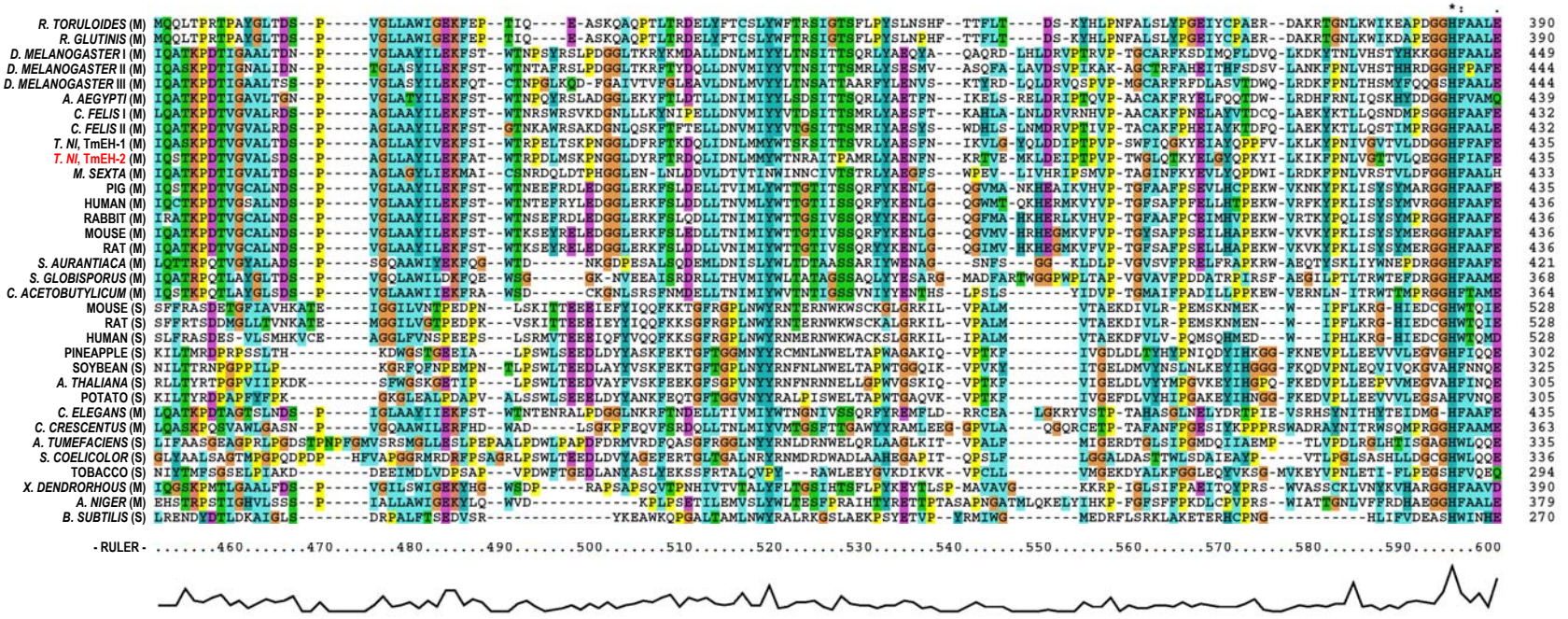
CLUSTAL X (v. 1.64b) amino acid sequence alignment used to construct the phylogenetic tree (Fig. 1.2) of microsomal (M) and soluble (S) epoxide hydrolases from a selection of organisms* spanning four kingdoms

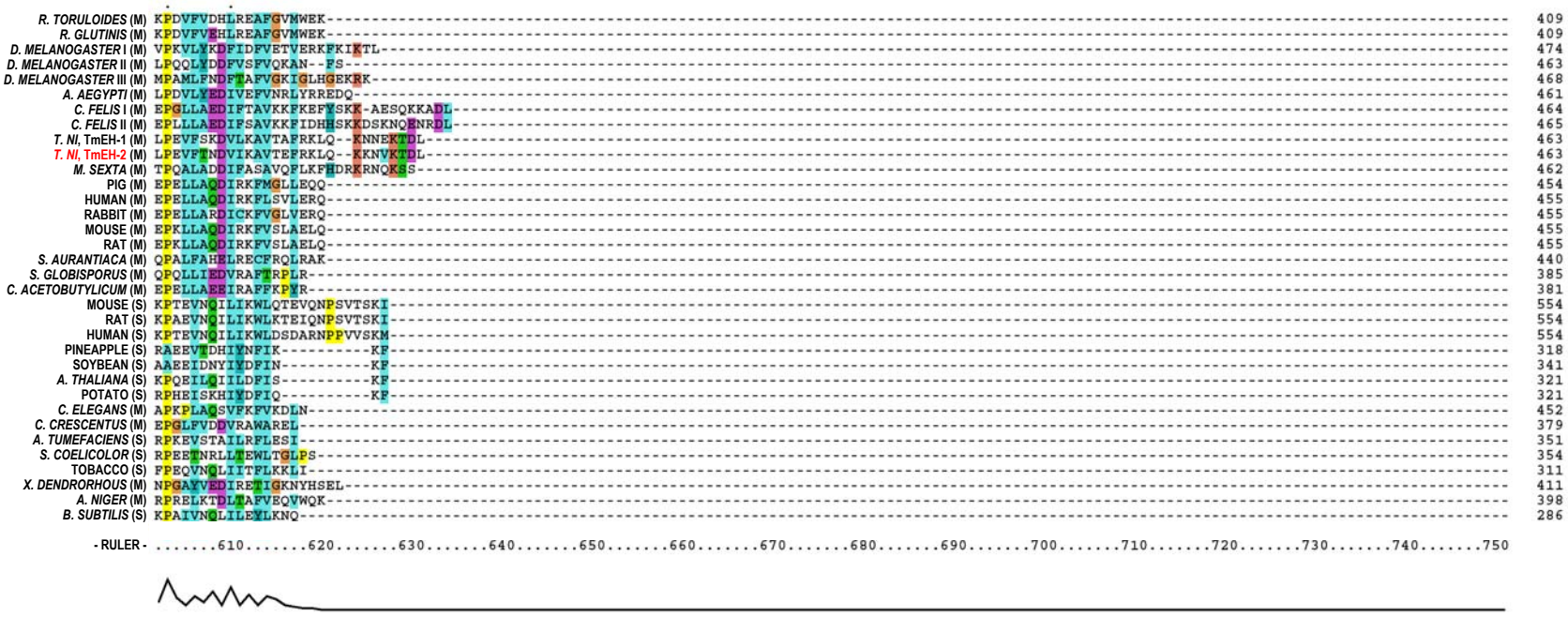
(Page 4 of 5)



* Complete scientific names and NCBI accession numbers of each organism are provided in Table 1.1.

CLUSTAL X (v. 1.64b) amino acid sequence alignment used to construct the phylogenetic tree (Fig. 1.2) of microsomal (M) and soluble (S) epoxide hydrolases from a selection of organisms* spanning four kingdoms

(Page 5 of 5)



* Complete scientific names and NCBI accession numbers of each organism are provided in Table 1.1.

APPENDIX B

ASSAYS FOR THE CLASSIFICATION OF TWO TYPES OF ESTERASES: CARBOXYLIC ESTER HYDROLASES AND PHOSPHORIC TRIESTER HYDROLASES

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Current Protocols in Toxicology
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ABSTRACT

Assay protocols are described that quantitate two types of esterases – the carboxylic ester hydrolases and the phosphoric triester hydrolases. The carboxylic ester hydrolases include the B-esterases, which are inhibited by organophosphorus compounds. Among the phosphoric triester hydrolases is aryldialkylphosphatase, which has been called A-esterase, or paraoxonase due to its ability to metabolize paraoxon and other organophosphates. These assays are colorimetric and miniaturized for rapid, simultaneous testing of multiple, small-volume samples in a microtiter plate format. A discussion follows on the history of esterase nomenclature, along with reasons why this large group of enzymes is so difficult to classify.

INTRODUCTION

Esterases (EC 3.1) are a large, heterogeneous group of enzymes classified under the general category of hydrolases (EC 3). Possessed by virtually all organisms, esterases hydrolyze a wide variety of substrates that contain ester linkages. It is well known that some esterases are involved in detoxication or activation of exogenous compounds, such as therapeutic drugs, anaesthetics and pesticides. Additionally, metabolism of certain chromogenic esters allow for esterase detection and quantitation. These enzymes also hydrolyze endogenous esters, although the substrates and physiological role have been difficult to determine.

This unit provides assay protocols to quantitate two common classes of esterases that are known to metabolize xenobiotics in a number of organisms. The first class is called the carboxylic ester hydrolases (CEHs) (EC 3.1.1). Within this group are the B-esterases, which are inhibited by organophosphates. The CEH assay (see Basic Protocol 1) colorimetrically measures endpoint metabolism of the substrate 1-naphthyl acetate to the product 1-naphthol (Fig. 1A). The other class of esterases to be assayed is the phosphoric triester hydrolases (PTEHs) (EC 3.1.8). Within this class is A-esterase, which also is referred to as paraoxonase due to its ability to metabolize the insecticide, paraoxon, as well as other organophosphates. The PTEH assay (see Basic Protocol 2) colorimetrically measures endpoint metabolism of the substrate, methyl paraoxon (dimethyl-*p*-nitrophenylphosphate), to the product *p*-nitrophenol (Fig. 1B).

Esterases are commonly found in serum and in both the microsomal and soluble fractions of cells. Therefore whole homogenates from tissues or cultured cells can be used in either assay after clarification by a single centrifugation (10,000 x *g*). A serum,

tissue or cell sample can be subjected to both protocols to determine the presence of one or both classes of esterases, and to quantitate respective enzyme activities. Alternatively, these assays can aid in the identification of a purified CEH or PTEH.

CAUTION: The Material Safety Data Sheet (MSDS) on methyl paraoxon labels this compound as "highly toxic". 1-Naphthol and phenyl thiourea is labeled "toxic", while Fast Blue B Salt and sodium dodecyl sulfate (SDS) are labeled "harmful". 1-Naphthyl acetate is labeled with "Caution". Handle these materials under a chemical fume hood when making stock and assay solutions. Investigators should access and read the MSDS of all chemicals prior to their use to be aware of hazards, proper handling procedures, and appropriate safety wear. At the end of experiments, extreme care should be taken to appropriately package, label, and temporarily store all chemical waste that is generated from performance of these protocols. Promptly contact the hazardous waste office of the institution for proper disposal instructions.

BASIC PROTOCOL 1

MEASUREMENT OF CARBOXYLIC ESTER HYDROLASE (CEH) ACTIVITY

This method measures hydrolysis of the carboxylic ester substrate, 1-naphthyl acetate to the product, 1-naphthol, in an endpoint assay. The product then forms a complex with Fast Blue B salt to yield a purple-blue color, which is detectable spectrophotometrically at 595 nm. Several concentrations of 1-naphthol are used to create a standard curve for estimation of CEH activity in samples. Multiple samples can be analyzed simultaneously, as the assay is formatted for a microtiter plate reader using 96-well plates. The procedure is rapid, with a total incubation time of 35 min. With a total reaction volume of 200 µl, only a small volume of sample is necessary.

Materials

100µmol/ml 1-Naphthol (see recipe)

200 mM 1-Naphthyl acetate (see recipe)

0.3% (w/v) Fast Blue B Salt solution containing 3.4% (w/v) SDS (see recipe)

100 mM methyl paraoxon, (see recipe)

Enzyme sample (purified protein or clarified homogenate)

100 mM Sodium phosphate buffer, pH 7.4 (see recipe)

Spectrophotometer for microtiter plates with temperature control

Plastic 96-well, flat-bottom microtiter plates

Prepare samples and solutions

1. Turn on the microtiter plate spectrophotometer and select the endpoint mode for reading absorbance at 595 nm. Also, set the incubator to the desired assay temperature.

   Samples from mammals, birds and E. coli normally are incubated at 37°C. An incubation temperature of 30°C may be more appropriate for fish, insect, reptile and plant samples.

2. From stock solutions, make fresh assay solutions of 1-naphthol (100, 50, 25, 12.5, 6.25, 3.13 nmol/ml), 1-naphthyl acetate (1 mM), and methyl paraoxon (10 mM). Use sodium phosphate assay buffer for these dilutions. Also, prepare a 0.3% (w/v) Fast Blue B Salt solution containing 3.4% (w/v) SDS in distilled water (see recipes).

3. Perform preliminary CEH assays with several dilutions of enzyme sample to determine an enzyme concentration that is within the linear range of substrate hydrolysis. Make sample dilutions in ice-cold sodium phosphate assay buffer.

   A regression of sample concentration versus absorbance (OD_{595}) should produce a straight line that intersects the origin in the linear range of the assay.

4. Prepare an adequate volume of the sample concentration in the linear range of the assay for addition (steps 8 and 9) of 70 µl to each well that requires enzyme sample. Use ice-cold sodium phosphate assay buffer for sample dilutions. Keep enzyme samples on ice before the addition of substrate.

CEH activity calculations may be inaccurate if the sample concentration assayed does not produce a linear rate of substrate hydrolysis.

Different sample preparations may vary significantly in CEH activity. If quantitating multiple samples, be aware that concentrations within the linear range of substrate hydrolysis for one sample may be different for other samples.

Avoid any delays during the execution of steps 5 to 15.

Perform CEH assay

5. For the standard blank, add 175 µl of sodium phosphate assay buffer to each of three empty wells of a 96-well microtiter plate.

6. For the standards, add 175 µl to each of three empty wells for each 1-naphthol concentration (3.13, 6.25, 12.5, 25, 50, and 100 nmol/ml).

7. For the sample blank, add 75 µl of sodium phosphate assay buffer to each of three empty wells.

   This blank will detect color formation from non-enzymatic sources.

   Do not confuse these sample blanks with the standard blanks (step 5). These two types of blanks differ in content.

8. For each sample, add the following in sequence to each of three empty wells:

   5 µl of sodium phosphate assay buffer

   70 µl of enzyme sample

9. For each sample plus methyl paraoxon treatment, add in sequence the following to each of three empty wells:

   5 µl of 10 mM methyl paraoxon

   70 µl of enzyme sample

   To calculate percentage inhibition, see CEH Activity Calculations below. Methyl paraoxon at the assay concentration should inhibit B-esterases (carboxylesterases, acetylcholinesterases and cholinesterases), which generally are in abundance in many types of samples.

   Additional sample wells can be included to evaluate other inhibitors or activators of CEH activity.

10. Immediately place the plate into the microtiter plate spectrophotometer and incubate for 10 min at the assay temperature.

    If the spectrophotometer has an automix function, enable it to shake-mix the contents of the plate wells at the beginning and end of each incubation period throughout the assay.

This incubation period allows for the methyl paraoxon to inhibit B-esterases.

11. Remove the plate from the spectrophotometer. Add 100 µl of 1.0 mM 1-naphthyl acetate substrate to all wells *except* those containing the standard blank and the standards.

12. Place the plate back into the spectrophotometer and incubate for 15 min at the assay temperature.

13. Remove the plate and add 25 µl of 0.3% Fast Blue B Salt solution containing 3.4% SDS to all wells of the assay.

14. Return the plate to the spectrophotometer and incubate for 10 min at the assay temperature.

    This incubation period allows for color development from the conjugation of naphthol-Fast Blue B.

Read Samples

15. Read simultaneously the absorbance of each well at 595 nm.

16. Remove the microtiter plate from the spectrophotometer. Appropriately package and label hazardous waste from the assay, and arrange for prompt removal and disposal by the hazardous waste office of the institution.

SUPPORT PROTOCOL 1

CEH SPECIFIC ACTIVITY CALCULATIONS

The following steps for activity calculations are based on acquisition of raw absorbance readings from the endpoint mode of a microtiter plate spectrophotometer. Spectrophotometers with accompanying computer and software may be programmable for automated blank subtraction, standard curve plotting and enzyme activity calculations.

1. Subtract the mean absorbance of the 3 standard blank wells from the absorbance of each well that contains 1-naphthol standards. Then calculate the mean absorbance (blank subtracted) of each standard concentration from the three replicate wells per concentration.

2. Create a standard curve by plotting each 1-naphthol concentration versus its corresponding absorbance.

   After plotting the standard curve, make sure that the best-fitted line passes through the origin of the graph. Also, the correlation coefficient (r^2 value) of the line typically should be ≥ 0.9. If necessary, the standard curve should be repeated until these criteria are met.

3. Subtract the mean absorbance of the three sample blank wells from the absorbance of each well that contains sample and sample plus methyl paraoxon.

   The blank-subtracted absorbance value of each sample well must be between the minimum and maximum OD_{595} values of the 1-naphthol standard curve. If

necessary, adjust the sample concentration and repeat the assay until the sample absorbance is in this range.

4. Use the linear regression equation of the standard curve to determine the concentration (nmol/ml) of 1-naphthol produced in each of the wells containing sample and sample plus methyl paraoxon.

   For example, with the equation y = mx + b, the concentration (x) can be determined by applying the blank-subtracted sample absorbance (y). The slope of the line is m, and b is the y-intercept.

5. Measure the protein concentration (mg/ml) of each sample.

6. Calculate the CEH specific activity for each well containing sample and sample plus methyl paraoxon using the following parameters: the concentration of 1-naphthol produced, the assay dilution factor of the enzyme sample, incubation time, and the protein concentration of the enzyme sample.

$$\frac{\text{nmol/ml of 1-naphthol X sample dilution factor}}{\text{incubation time (min)}} \text{ X } \frac{\text{1 ml}}{\text{mg protein}} = \frac{\text{nmol min}^{-1}}{\text{mg protein}^{-1}}$$

7. For each sample and sample plus methyl paraoxon treatment, calculate the mean specific activity of CEH from the three replicate wells per treatment.

8. To calculate the percentage of CEH inhibition by methyl paraoxon, use the following equation:

$$\frac{\text{(specific activity of sample)} - \text{(specific activity of sample + methyl paraoxon)}}{\text{(specific activity of sample)}} \times 100$$

BASIC PROTOCOL 2

MEASUREMENT OF PHOSPHORIC TRIESTER HYDROLASE (PTEH) ACTIVITY

This method measures hydrolysis of the phosphoric triester substrate, methyl paraoxon. The yellow product, *p*-nitrophenol is detectable spectrophotometrically at 405 nm. Several concentrations of *p*-nitrophenol are used to create a standard curve for estimation of PTEH activity in samples. Multiple samples can be analyzed simultaneously, as the assay is formatted for a microtiter plate reader using 96-well plates. This procedure is rapid, with a total incubation time of 45 min. With a total reaction volume of 300 µl, only a small volume of sample is necessary.

Materials

100 µmol/ml *p*-nitrophenol (see recipe)

100 mM methyl paraoxon (see recipe)

Enzyme sample (purified or clarified homogenate)

100 mM Tris-HCl assay buffer, pH 8.0 (see recipe)

Spectrophotometer for microtiter plates with temperature control

Plastic 96-well, flat-bottom microtiter plates

Prepare samples and solutions

1. Turn on the microtiter plate spectrophotometer and select the endpoint mode for reading absorbance at 405 nm. Also, set the incubator to the desired assay temperature.

Samples from mammals, birds and E. coli normally are incubated at 37°C. An incubation temperature of 30°C may be more appropriate for fish, insect, reptile and plant samples.

2. From stock solutions, make fresh assay solutions of *p*-nitrophenol (100, 50, 25, 12.5, 6.25, 3.13 nmol/ml), and methyl paraoxon (2 mM). Use Tris-HCl assay buffer for these dilutions.

3. Perform preliminary PTEH assays with several dilutions of enzyme sample to determine an enzyme concentration that is within the linear range for substrate hydrolysis. Make sample dilutions in ice-cold Tris-HCl assay buffer.

   A regression of sample concentration versus absorbance (OD_{405}) should produce a straight line that intersects the origin in the linear range of the assay.

4. Prepare an adequate volume of the sample concentration in the linear range of the assay for addition (step 8) of 289 µl to each well that requires enzyme sample. Use ice-cold Tris-HCl buffer for sample dilutions. Keep enzyme samples on ice before the addition of substrate.

   PTEH activity calculations may be inaccurate if the sample concentration assayed does not produce a linear rate of substrate hydrolysis.

   Different sample preparations may vary significantly in PTEH activity. If quantitating multiple samples, be aware that concentrations within the linear range of substrate hydrolysis for one sample may be different for other samples.

Avoid any delays during the execution of steps 5 to 10.

Perform PTEH assay

5. For the standard blank, add 300 µl of 100 mM Tris-HCl assay buffer to each of three empty wells of a 96-well microtiter plate.

6. For the standards, add 300 µl to each of three empty wells for each *p*-nitrophenol concentration (3.13, 6.25, 12.5, 25 and 100 nmol/ml).

7. For the sample blank, add the following to each of three empty wells of the microtiter plate:

   11 µl of 2 mM methyl paraoxon

   289 µl of 100 mM Tris-HCl assay buffer

   This blank will measure the non-enzymatic rate of substrate hydrolysis.

   Do not confuse sample blanks with the standard blanks (step 5). These two types of blanks differ in content.

8. For each SAMPLE, add the following to each of 3 empty wells of the microtiter plate:

   11 µl of 2 mM methyl paraoxon

   289 µl of enzyme sample

9. Immediately place the microtiter plate into the spectrophotometer and incubate for 45 min at the assay temperature.

   If the spectrophotometer has an automix function, enable it to shake-mix the contents of the plate wells at the beginning and end of the incubation period.

   Within the first few minutes of incubation, a fraction of methyl paraoxon will bind and inhibit B-esterases. The remainder of the organophosphate then acts solely as a substrate for PTEH.

Read samples

10. Read simultaneously the absorbance of each well at 405 nm.

11. Remove the microtiter plate from the spectrophotometer. Appropriately package and label hazardous waste from assay, and arrange for prompt removal and disposal by the hazardous waste office of the institution.

SUPPORT PROTOCOL 1

PTEH SPECIFIC ACTIVITY CALCULATIONS

The following steps for activity calculations are based on acquisition of raw absorbance readings from the endpoint mode of a microtiter plate spectrophotometer. Spectrophotometers with accompanying computer and software may be programmable for automated blank subtraction, standard curve plotting and enzyme activity calculations.

1. Subtract the mean absorbance of the three standard blank wells from the absorbance of each well that contains *p*-nitrophenol standards. Then calculate the mean absorbance (blank subtracted) of each standard concentration from the three replicate wells per concentration.

2. Create a standard curve by plotting each *p*-nitrophenol concentration versus its corresponding absorbance.

   After plotting the standard curve, make sure that the best-fitted line passes through the origin of the graph. Also, the correlation coefficient (r^2 value) of the line typically should be ≥ 0.9. If necessary, the standard curve should be repeated until these criteria are met.

3. Subtract the mean absorbance of the three sample blank wells from the absorbance of each well that contains sample.

   The blank-subtracted absorbance value of each sample well must be between the minimum and maximum OD_{405} values of the p-nitrophenol standard curve. If

necessary, adjust the sample concentration and repeat the assay until the sample absorbance is in this range.

4. Use the linear regression equation of the standard curve to determine the concentration (nmol/ml) of *p*-nitrophenol produced in each of the wells containing sample.

   For example, with the equation y = mx + b, the concentration (x) can be determined by applying the blank-subtracted sample absorbance (y). The slope of the line is m, and b is the y-intercept.

5. Measure the protein concentration (mg/ml) of each sample.

6. Calculate the PTEH specific activity for each well containing sample using the following parameters: the concentration of *p*-nitrophenol produced, the assay dilution factor of the enzyme sample, incubation time, and the protein concentration of the enzyme sample:

$$\frac{\text{nmol/ml of } p\text{-nitrophenol} \times \text{sample dilution factor}}{\text{incubation time (min)}} \times \frac{1 \text{ ml}}{\text{mg protein}} = \frac{\text{nmol min}^{-1}}{\text{mg protein}^{-1}}$$

7. For each sample, calculate the mean specific activity of PTEH from the three replicate wells.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps.

Fast Blue B Salt solution, 0.3% (w/v) containing 3.4% (w/v) SDS

In a foil-covered container, dissolve 30.0 mg of Fast Blue B Salt (~90%; Aldrich) in distilled water for a total volume of 3.0 ml. In a separate container, add 340.0 mg of SDS (sodium dodecyl sulfate salt) in distilled water for a total volume of 7.0 ml. To dissolve the SDS, gently swirl its container by hand to minimize foaming. When both chemicals are dissolved, add the SDS solution to the Fast Blue B Salt solution container. Mix by gentle hand swirling. This solution is stable for ~ 2 hr.

Fast Blue B Salt is light sensitive and must be kept in darkness except when weighing, and during addition of this solution to the assay.

CAUTION*: SDS is a respiratory irritant. A dust mask should be worn during weighing and mixing of SDS.*

Methyl Paraoxon, 100 mM

Stock solution: Add 24.71 mg of methyl paraoxon (99.1%; Chem Service) to 100% ethanol for a final volume of 1.0 ml. Vortex until dissolved. Store in a tightly sealed container for up to 1 month at 4^{o}C.

Inhibitor solution for CEH assay (10 mM): Make fresh daily by diluting the stock solution with 100 mM sodium phosphate assay buffer, pH 7.4 (see recipe).

Substrate solution for PTEH assay (2 mM): Make fresh daily by diluting the stock solution with 100 mM Tris-HCl assay buffer, pH 8.0 (see recipe).

CAUTION*: Methyl paraoxon is highly toxic! Appropriate protective gloves and eyewear should be worn, and a chemical fume hood should be used when making and diluting solutions.*

1-Naphthol, 100 µmol/ml

Stock solution: Add 14.42 mg of 1-naphthol (99+%; Aldrich) to 100% ethanol for a total volume of 1.0 ml. Vortex until dissolved. Store in a tightly sealed container for up to 1 month at –20°C.

CEH standard assay solutions (100, 50, 25, 12.5, 6.25 and 3.13 nmol/ml): Make fresh daily by diluting the stock solution with 100 mM sodium phosphate assay buffer, pH 7.4 (see recipe).

1-Naphthyl Acetate, 200 mM

Stock solution: Add 37.24 mg of 1-naphthyl acetate (99+%; Sigma) to 100% ethanol for a total volume of 1.0 ml. Vortex until dissolved. Store in a tightly sealed container for up to 1 month at -20°C.

CEH substrate assay solution (1 mM): Make fresh daily by diluting the stock solution with 100 mM sodium phosphate assay buffer, pH 7.4 (see recipe).

***p*-Nitrophenol, 100 μmol/ml**

Stock solution: Add 13.91 mg of *p*-nitrophenol (99%; Fluka Chemical) to 100% ethanol for a total volume of 1.0 ml. Vortex until dissolved. Store in a tightly sealed container for up to 1 month at –20°C.

PTEH standard assay solutions (100, 50, 25, 12.5, 6.25 and 3.13 nmol/ml): Make fresh daily by diluting the stock solution with 100 mM Tris-HCl assay buffer, pH 8.0 (see recipe).

Sodium phosphate assay buffer, 100 mM (pH 7.4)

Monobasic stock solution, 1 M: Add 11.996 g of monobasic sodium phosphate (NaH_2PO_4) to distilled water for a total volume of 100 ml. Heat to dissolve if necessary. Store for up to 2 months at 4°C. Heat to re-dissolve if precipitation occurs.

Dibasic stock solution, 1 M: Add 14.196 g of dibasic sodium phosphate (Na_2HPO_4) to distilled water for a total volume of 100 ml. Heat to dissolve if necessary. Store for up to 2 months at 4°C. Heat to re-dissolve if precipitation occurs.

Sodium phosphate assay buffer, 100 mM (pH 7.4): At 25°C, add 2.26 ml of 1 M monobasic stock solution and 7.74 ml of 1 M dibasic stock solution to 90 ml of distilled water. Mix thoroughly and measure to assure a pH of 7.4. If pH is not

7.4, remake assay buffer with new monobasic and dibasic stock solutions. Store for up to 2 months at 4°C.

For homogenization of insects, add phenyl thiourea (PTU) to the assay buffer. Under a chemical fume hood, heat-dissolve the PTU in a small volume of water, and then add to the assay buffer for a concentration of 0.01% (w/v). PTU will prevent blackening of the sample by inhibiting tyrosinases.

Tris-HCl assay buffer, 100 mM (pH 8.0)

Dissolve 1.211g of Tris base in distilled water for a total volume of 80 ml. Adjust the pH to 8.0 with concentrated HCl. Bring total volume to 100 ml with distilled water. Store up to 1 month at 4°C.

For homogenization of insects, add phenyl thiourea (PTU) to the assay buffer. Under a chemical fume hood, heat-dissolve the PTU in a small volume of water, and then add to the assay buffer for a concentration of 0.01% (w/v). PTU will prevent blackening of the sample by inhibiting tyrosinases.

COMMENTARY

Background Information

Esterases are monomeric (mol. wt. 40,000 to 60,000) or heteromeric (mol. wt. $\leq$ 200,000) proteins present in the soluble and microsomal cell fractions. Some may have post-translational modifications, including glycosylation and the addition of lipids (Urich, 1994). The types of esterases present and levels of activity can be highly variable from one species to another, and may differ even between individuals of the same species. Several factors can influence these differences, including life stage, hormones, sex, diet, food quality, genetic polymorphism, disease, and environmental conditions. Some esterases are associated with specific tissues, while others are ubiquitous within an organism (Devorshak and Roe, 1998).

The esterases cleave aliphatic esters of short-chain carboxyl acids, aromatic esters, aromatic amides, phosphoesters, and thioesters. The physiological function of many esterases is still obscure, but they probably are essential because their genetic codes have been preserved throughout evolution (Van Zutphen et al., 1988; Urich, 1994). Several studies have suggested that human serum esterases are involved in metabolizing various classes of lipids, such as mono- and triacylglycerols (Tsujita and Okuda, 1983; Shirai et al., 1988). Accumulation of such lipids may be caused by poor esterase metabolism, which has been implicated in the development of human athersclerosis and myocardial infarction (McElveen et al., 1986; Mackness, 1989). A well known endogenous esterase substrate is acetylcholine, which is specifically metabolized by acetylcholinesterase in nerve tissue for proper impulse transmission. In insects, an esterase has been discovered that is highly specific for the endogenous substrate, juvenile hormone (JH). JH esterase metabolism of JH at critical time points is essential for proper

insect development and metamorphosis (Roe et al., 1993). In general, however, evidence of specific endogenous substrates for esterases is scarce.

Numerous exogenous esterase substrates, including xenobiotics, have been identified. Typically, esterase metabolism of xenobiotics is a detoxication mechanism that produces a less toxic, more water-soluble compound for excretion. In some cases however, the parent compound is activated by esterases, rendering higher toxicity to the intermediates or final products. Examples of exogenous substrates include ester and amide derivatives of drugs, nerve gases (tabun, sarin and DFP), plasticizers that contain phthalic acid esters, herbicides with phenoxyacetic and picolinic acid esters, and several classes of insecticides (Hodgson and Levi, 1994). Development of resistance in insects to the organophosphate, pyrethroid, and carbamate insecticides has been attributed to esterases. The mechanism of resistance usually is associated with increased esterase production, which enhances detoxication or sequestration of the insecticides (see Devorshak and Roe, 1998).

In 1953, Aldridge proposed a classification system of esterases based upon their interaction with organophosphorus compounds (OPs). "A"-esterases hydrolyze OPs like paraoxon, methyl paraoxon, and diisopropylphosphorofluoridate (DFP), while "B"-esterases are inhibited by OPs. With A- and B-esterases, the OPs are substrates for the enzyme. But in the case of the B-esterases, these compounds act as suicide substrates by binding their phosphoryl moieties to the enzymes (Walker, 1989).

Since the time of Aldridge's discovery of A- and B-esterases, hundreds of types of esterases have been discovered. The Enzyme Commission of the International Union of Biochemistry and Molecular Biology (IUBMB, 1992) has classified these esterases based upon their metabolic activities toward a variety of artificial substrates. This

classification system has been criticized heavily for several reasons (Pen and Beintema, 1986; Urich, 1994). First, artificial substrates are used, since neither the endogenous substrate nor the biological function is known for most esterases. Second, substrate preferences can be highly overlapping between the defined classes. Third, many esterases exist in multiple forms (isozymes) due to genetic polymorphism, or as a result of variable glycosylation and other post-translational modifications. Fourth, other enzymes have esterase activity, such as serine proteases. Also, proteins that usually are not considered to be enzymes, like serum albumins, can have esterase activity. Finally, only a few esterases have been purified extensively for characterization. When working with unpurified homogenates, substrate metabolism may result from more than one type of esterase, or perhaps from a protein other than an esterase. Because of these pitfalls, the classification system used today lends itself to problems of ambiguity and redundancy. Ideally the classification of esterases would be similar to that of enzymes such as the cytochrome P450s, which is based on a genealogical tree constructed from the relativity of nucleic acid and peptide sequences. Unfortunately molecular genetics data on esterases are insufficient at this time to derive such nomenclature. Until the volume of molecular data is adequate to develop this type of classification system for esterases, suggestions for improvement (e.g. Heymann 1989; Walker 1989) of the current system should be considered and implemented when deemed appropriate.

Despite the difficulties and controversy associated with the identification and classification of esterases in the past, Aldridge's scheme still is used today to distinguish and quantitate esterases that metabolize OPs, and those that are inhibited by these compounds. However, the methodology of detecting these esterases, and the nomenclature (discussed below) has changed significantly. Originally, Aldridge used *p*-

nitrophenyl esters (acetate, propionate and butyrate) as substrates in a manometric, rather than a colorimetric method. Enzyme activity was estimated kinetically, based on the CO_2 liberated from bicarbonate buffer by the acid produced during hydrolysis (Aldridge, 1953). A- and B-esterases were distinguished by the difference in activity between reactions with, and without paraoxon. The disadvantages of this assay include a tedious protocol, low sensitivity of detection, and use of large sample volumes. Also, it was time consuming since only one sample could be measured at a time. The esterase protocols presented here have the advantages of being colorimetric and miniaturized for multi-well microtiter plates. This simpler format allows for rapid, simultaneous quantitation of multiple samples. Additionally, these assays are very sensitive and require less sample material. Other colorimetric esterase assays have been published previously (see below), but these macroassay procedures utilized large sample volumes and were limited to one sample reading at a time.

The first assay presented (Basic Protocol 1) is for the measurement of carboxylic ester hydrolases (CEHs; EC 3.1.1). Currently there are 66 subclasses of enzymes within this group, including the B-esterases, which are inhibited by OPs. B-esterases include carboxylesterase (EC 3.1.1.1), acetylcholinesterase (EC 3.1.1.7), and cholinesterase (EC 3.1.1.8). Besides the B-esterases, there is a wide variety of other CEHs, such as arylesterase (EC 3.1.1.2), sterol esterase (EC 3.1.1.13), α-amino acid esterase (EC 3.1.1.43), insect juvenile hormone esterase (EC 3.1.1.59), and several lipases and lactonases. Originally, the assay for CEHs was miniaturized and modified by Abdel-Aal et al. (1990) from macroassays by Gomori (1953) and van Asperen (1962). This procedure measures hydrolysis of the substrate, 1-naphthyl acetate, to the product, 1-naphthol. Although some researchers consider 1-naphthyl acetate to be specific for

carboxylesterases, the protocol presented here is titled more generally due to the possibility that other CEHs can metabolize this substrate. To determine if metabolism of 1-naphthyl acetate was a result of B-esterases, esterase activity can be quantitated from samples with, and without the addition of methyl paraoxon.

The second protocol described (Basic Protocol 2) is for detection of phorphoric triester hydrolases (PTEHs; EC 3.1.8). Currently there are only two subclasses of esterases in this group. Besides A-esterase, now called aryldialkylphosphatase (EC 3.1.8.1) by the IUBMB, the other member is diisopropyl-fluorophosphatase (EC 3.1.8.2), or DFP-ase. Both of these enzymes metabolize OPs, including esters of phosphonic and phosphinic acids, and those with phosphorus anhydride bonds (IUBMB, 1992). The assay is a modification of a microtiter plate procedure developed by Devorshak and Roe (2001), which is based on a macroassay described by Furlong et al. (1988). The substrate is methyl paraoxon, which when metabolized by PTEHs, yields the yellow colored product, *p*-nitrophenol. The assay discriminates against B-esterases, as this activity is inhibited by the organophosphorus substrate.

Several inhibitors and activators of PTEH activity have been documented (Aldridge 1953; Shishido and Fukami, 1972; Konno et al., 1990; Devorshak and Roe 2001). Perhaps the most effective PTEH inhibitors are mercuric compounds such as *p*-chloromercuribenzoate (PCMB). Other metal ions such as Ag^{+}, Cd^{2+}, Cu^{2+} and Sn^{4+} can also inhibit PTEHs, as well as PO_4 and ethylenediaminetetraacetic acid (EDTA). In general, these inhibitors are not as potent as mercury, and results differ widely among various biological samples. PTEH activity may be enhanced by addition of Ca^{2+}, Co^{2+} and Mn^{2+}. These ions act as important cofactors for PTEH activity, and may be present naturally in samples from serum or whole homogenates from cells or tissues. Purified

PTEH samples, however, may require addition of one or more of these cofactors to elicit activity. In mammals, Ca^{2+} appears to be the most important cofactor, while Co^{2+} and Mn^{2+} activate insect PTEH activity (Dauterman 1976). Inhibition of PTEH by Hg^{2+} and the metal chelator EDTA, and activation by divalent cations suggests that PTEH may have a cysteine residue involved in the catalytic mechanism. This is not the case for the B-esterases, which have a serine at the active site of the enzyme (Aldridge 1989).

Utilizing both protocols, one can determine quickly if biological samples contain CEHs, PTEHs, or both types of esterases. Furthermore, esterase activity can be detected in the pmol min^{-1} mg $protein^{-1}$ range. With the miniaturized microtiter plate design of these assays, multiple, small-volume samples can be tested simultaneously. Finally, additional treatments can be added to either assay for screening of classical or putative inhibitors or activators of these esterases.

Critical Parameters and Troubleshooting

Sample preparation

Small particles suspended in the supernatants of sample homogenates following centrifugation (10,000 x *g*) can cause erroneous absorbance readings. This suspended material can be eliminated by filtration of supernatants through glass wool. The glass wool should be cleaned with HPLC grade hexane and then air-dried before use. Supernatants of samples can be assayed fresh or can be stored at -20^{o} to -80^{o}C until the time of assay. When freezing, make small aliquots of each sample to avoid repetitive freeze-thawing which could decrease enzyme activity. Always hold samples on ice immediately after thawing and until addition to the microtiter plate.

It is advisable to include in all assays a positive control sample (if available) that is known to have detectable activity of the esterase of interest. Modifications to the assay protocol may be necessary if activity is detectable in the positive control, but not in an unknown sample. For example, increasing sample concentration or incubation time may be necessary to detect low levels of activity. Additionally, some esterases are more active at slightly higher or lower pH than is listed in the protocol. Samples can be assayed at a range of pH values to determine optimal conditions for esterase activity. If the adjustment of assay conditions does not elicit detectable enzyme activity, it may be possible that the sample either has no activity, or it is too low for detection.

Assay conditions

For CEH and PTEH assays, enzyme activity measurements will be inaccurate if the enzyme concentration is not within the linear range of substrate hydrolysis. As described in Basic Protocols 1 and 2, preliminary assays may be necessary to identify an appropriate enzyme concentration.

The substrates, 1-naphthyl acetate and methyl paraoxon, are considered to be specific for carboxylic ester hydrolases and phosphoric triester hydrolases, respectively. Despite this consideration, it should be realized that there is the possibility for the substrates to be metabolized by other enzymes.

Whenever inhibitors or activators are added to the assays in a solvent, rather than an aqueous diluent, the total percentage of solvent (including substrate addition) in the final reaction volume should never exceed 1.0%. Higher solvent percentages could significantly affect enzyme activity.

When performing CEH assays, potassium phosphate should not be substituted for sodium phosphate buffer. Potassium will precipitate upon the addition of Fast Blue B-SDS solution in the assay.

Concerning the PTEH assay, there are several parameters that are critical for successful activity quantitation. Perhaps most important is proper pH of the enzymatic reactions. If the assay pH is too basic, high background may result from non-enzymatically hydrolyzed methyl paraoxon. If the assay pH is too acidic, the *p*-nitrophenol product loses its yellow color, and PTEH activity will not be detectable. For ideal activity measurements with low background, the assay should be pH 8.0-8.5. PTEH activity is highest at this pH range in mammals and insects. Also, the use of Tris-HCl buffer (pH 8.0) is highly recommended for sample preparation and assay reactions in PTEH assays. Other buffers may have constituents that affect PTEH activity. For instance, buffers containing phosphate ions or ethylenediaminetetraacetic acid (EDTA) can reduce or even fully inhibit PTEH activity.

Anticipated Results

Mouse liver, porcine liver, and the tobacco budworm, *Heliothis virescens* (whole body, 5^{th} stadium larva) were analyzed for CEH (Table 1) and PTEH (Table 2) specific activity by following the two protocols above. Whole homogenates were prepared from mouse liver and tobacco budworm in Tris-HCl buffer by use of a Polytron PT10/35 homogenizer (Brinkman Instruments). The homogenates were centrifuged for 15 min at 10,000 x *g*, and then filtered through glass wool. The porcine liver (41 U/mg) was ordered as a crude lyophilized powder from Sigma Chemical, and was resuspended in Tris-HCl assay buffer, pH 8.0. Samples were diluted in sodium phosphate assay buffer,

pH 7.4, for CEH assays and Tris-HCl assay buffer, pH 8.0, for PTEH assays. Protein concentrations were determined by the Bio-Rad assay with bovine serum albumin as the standard.

Basic protocol 1

See the CEH protocol for general assay requirements. Assays were performed at pH 7.4, and incubated with substrate for 15 min. The incubation temperature for mouse liver and porcine liver was 37^{o}C, while the tobacco budworm sample was incubated at 30^{o}C. Protein concentrations used for assays were 180, 382 and 37 μg for mouse, porcine, and tobacco budworm, respectively. For inhibitor treatments, methyl paraoxon (667 μM final assay concentration) was added to the sample for a 10-min incubation before addition of substrate. The specific activity (nmol min^{-1} mg protein^{-1}) and % inhibition by methyl paraoxon for each sample were calculated using the formulae in the CEH Calculations (see Support Protocol 1). See Table 1 for results.

Basic protocol 2

See the PTEH protocol for general assay requirements. Assays were performed at pH 8.0, and incubated with substrate for 45 min. The incubation temperature for mouse liver and porcine liver was 37^{o}C, while the tobacco budworm sample was incubated at 30^{o}C. Protein concentrations used for assays were 2.30, 0.03 and 3.81 μg for mouse, porcine and tobacco budworm, respectively. The specific activity (pmol min^{-1} mg protein^{-1}) for each sample was calculated using the formula in the PTEH Calculations (see Support Protocol 2). See Table 2 for results.

Time Considerations

For both assays, the total time required to perform the reactions depends on the number of samples and inhibitors to be analyzed. If adequate activity is detected with the incubation times listed for each protocol, the CEH assay can be performed in 45-60 min, while the PTEH assay may take 60-75 min. This time estimation does not include the preparation of buffers, standards, substrates or enzyme dilutions.

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Key References

Abdel-Aal et al., 1990. See above.

Describes, in detail, the carboxylic ester hydrolase assay (called 1-naphthyl acetate esterase assay in that publication) that was modified and miniaturized for microtiter plate format. A modification of this procedure is presented in Basic Protocol 1.

Aldridge, 1953. See above.

Original description of A- and B-esterase distinction.

Devorshak and Roe, 2001. See above.

Describes, in detail, the phosphoric triester hydrolase assay that was miniaturized for microtiter plate format. This procedure was modified (Basic Protocol 2) to provide endpoint absorbance data.

Table 1. Results from Carboxylic Ester Hydrolase (CEH) Assay

	CEH Activity (nmol min^{-1} mg $protein^{-1}$)	% Inhibition by Methyl Paraoxon[a]
Mouse Liver	171	100
Porcine Liver	13,713	99
Tobacco Budworm	64	98

[a]Final assay concentration of 667 μM.

Table 2. Results of the Phosphoric Triester Hydrolase (PTEH) Assay

	PTEH Activity (pmol min^{-1} mg $protein^{-1}$)
Mouse Liver	42
Porcine Liver	5
Tobacco Budworm	100

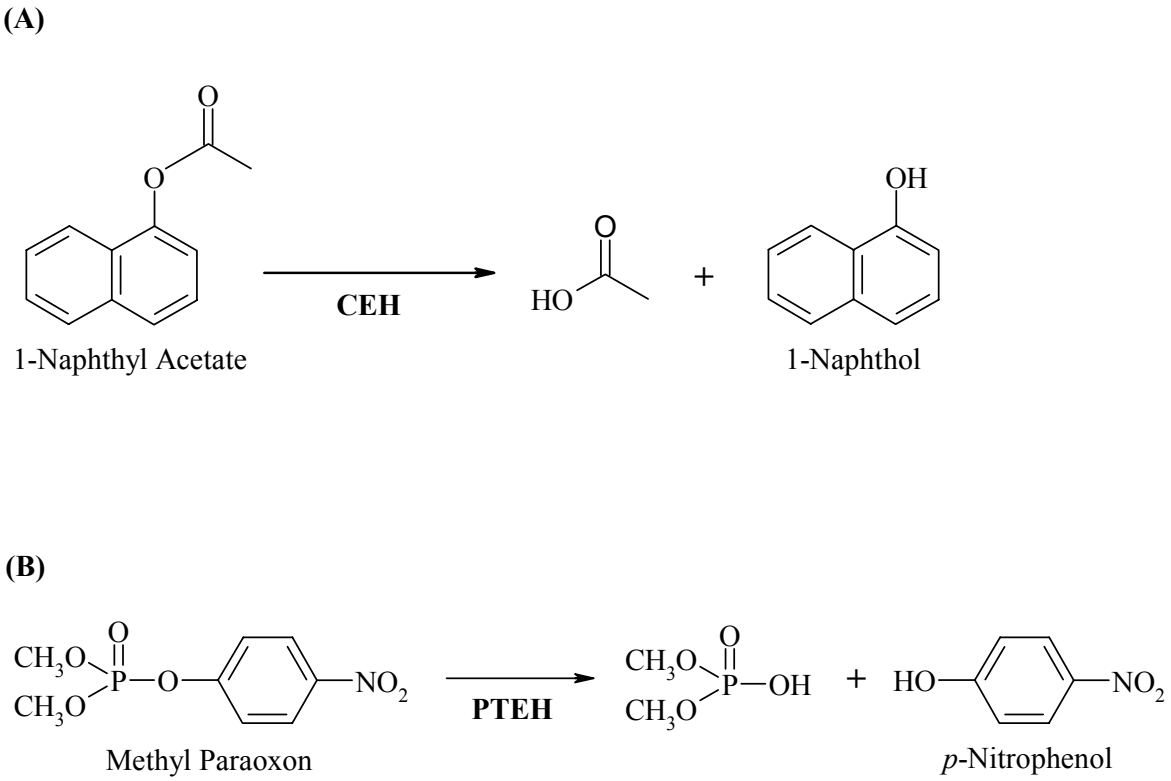

Figure 1. (A) Metabolism of 1-naphthyl acetate by carboxylic ester hydrolases (CEHs). (B) Metabolism of methyl paraoxon by phosphoric triester hydrolases (PTEHs).