2005 Beltwide Cotton Conferences, New Orleans, Louisiana - January 4 - 7, 2005

MOLECULAR CHARACTERIZATION OF EPOXIDE HYDROLASE CDNAS FROM THE CABBAGE LOOPER, TRICHOPLUSIA NI

Douglas D. Anspaugh, Sayed M. Khalil, Michael D. Tomalski, Deborah M. Thompson and R. Michael Roe
North Carolina State University
Raleigh, NC

Abstract

A full-length epoxide hydrolase (EH) cDNA (TmEH-2) and a partial EH cDNA (TmEH-3) were isolated from a *Trichoplusia ni* digestive system cDNA library made with equal proportions of fifth stadium, day 1 (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. TmEH-2 differed from a *T. ni* EH cDNA (TmEH-1) previously cloned from fat body. TmEH-2 was 67 and 73% identical at the nucleic acid and amino acid levels, respectively, to TmEH-1. A portion (789 nt) of TmEH-3 was sequenced yielding 293 amino acids in one open reading frame by conceptual translation. The nucleotide sequence of TmEH-3 was 64.9 and 65.4% identical to TmEH-1 and TmEH-2, respectively. The deduced amino acid sequence was 73.8% similar (with 63.2% identity) and 75.7% similar (with 66.2% identity) to TmEH-1 and TmEH-2, respectively.

TmEH-1 was subcloned into a baculovirus system for *in vivo* expression in *T. ni* larvae. Following injections of the non-occluded TmEH-1 virus, no increased JH III epoxide hydrolase activity was found compared to larvae injected with non-transformed control baculovirus. 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 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. In mammals, a large number of reports are available on the role of EHs in the metabolism of xenobiotic (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 (Brooks et al., 1970; Brooks, 1974 and 1977; Slade et al., 1975; Mullin, 1988; Taniai et al., 2003). 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) (Halarnkar et al., 1993; Kallapur et al., 1996; Harris et al., 1999 and others reviewed by Hammock, 1985; Roe and Venkatesh, 1990; and Gilbert et al., 2000). Although the role of the different EHs in endogenous and xenobiotic metabolism is not yet clear, Taniai et al., (2003) found that a clofibrate-inducible microsomal EH from *D. melanogaster* metabolized cis-stilbene epoxide but not JH III.

Several reports have been published describing insect EH cDNAs from *Manduca sexta* (Wojtasek and Prestwich, 1996), *Ctenocophalides felis* (Keiser et al., 2002), and *Trichoplusia ni* (Harris et al., 1999; Anspaugh, 2003). cDNAs from *Drosophila melanogaster, Aedes aegypti, Anopheles gambiae, Apis mellifera* and *Bombyx mori* are available from the GenBank database. Multiple EH alleles in insects were suggested by Mullin (1988) and validated in studies with *D. melanogaster* (3 EH cDNAs identified) and *C. felis* (2 EH cDNAs). All of the epoxide hydrolases sequenced from insects to date have been microsomal enzymes (reviewed by Anspaugh, 2003).

In the present study we report the cloning and sequencing of a second, unique EH cDNA (TmEH-2; NCBI accn. AF035482) and of a portion of a third EH cDNA (TmEH-3; NCBI accn. BG354599) from the digestive system of *T. ni*. 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 (Department of Entomology, University of California, Riverside). Larvae were reared on an artificial diet (Roe et al., 1982) at 27 ± 1°C with a relative humidity of 50 ± 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 (TmEH-2)

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 accn. U35736), a 237 base pair (bp) putative EH cDNA fragment isolated by RT-PCR of total RNA from L5D3 *T. ni* (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 of 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 labeled 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 accn. AF035482) in pGUT-EH is referred to as TmEH-2.

cDNA library screening (TmEH-3)

One hundred cDNAs were randomly chosen from the gut library described above. White (recombinant) plaques were subjected to PCR to determine the length of the cDNA insert contained in the isolated phage. Each plaque was picked separately using a sterile pasture pipette and transferred into a 0.5 ml tube containing 50µl sterile SM buffer. These were stored at 4°C overnight to allow phage to enter the SM buffer. One-half µl of phage in SM buffer was added to 29 µl of sterile water and frozen at -20°C for at least 1 h to break the phage coat. PCR was conducted using the

universal T3 and T7 primers (50 pmol each), 1x reaction buffer, 200µM each dNTP, and 2 units *Taq* DNA Polymerase (Promega). PCR conditions were 95°C for 5 min followed by 35 cycles each containing 95°C for 1 min, 40°C for 1 min and 72°C for 2 min. Following the last cycle there was a final extension of 72°C for 7 min. Five µl of each reaction were analyzed using agarose gel electrophoresis. Amplification products of 1000 bp or larger were sequenced, and the phage yielding these amplification products were subjected to single clone excision according to the instructions of the Zap Express cDNA Synthesis and Gigapack II Gold Cloning kit (Stratagene) and stored at -80°C. Following sequencing to yield expressed sequence tags (ESTs; see below) the resulting DNA sequences were compared with sequences in the NCBI GenBank database by the Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov; National Institutes of Health, Bethesda, MD) to determine the putative function of each EST. One clone, SK59, was similar to epoxide hydrolases and therefore chosen for further sequencing and analysis.

DNA sequencing and sequence analysis (TmEH-2)

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 a putative epoxide hydrolase by BLAST. The amino acid sequence was obtained by computational translation of the EH cDNA using the 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 cDNA or protein sequences.

DNA sequencing and sequence analysis (TmEH-3)

PCR products were purified using the QIAquick PCR Purification kit (QIAGEN, Valencia, CA). Purified PCR products were sequenced using the ABI PRISM® BigDye™ Terminators Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reactions contained 3 pmol of T3 primer, 50 ng of purified PCR product and 8µl BigDye terminator mix in a total reaction volume of 20µl. The thermal cycling conditions were 35 cycles at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. The reaction mix was then purified using a CENTRI-SEP column (Princeton Separation, Adelphia, NJ). The purified PCR product was dried in a vacuum centrifuge and sent to the Forest Biotechnology lab at North Carolina State University to be analyzed on ABI PRISM 370 DNA Analyzer (Applied Biosystems).

TmEH-1 baculovirus expression *in vivo* in *T. ni* larvae

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 at the base of the second pair of prolegs (from the anterior) of L5D0 *T. ni*. Uninfected 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 measured with a Hamilton syringe (Reno, NV) and then aspirated and delivered by mouth inhalation and exhalation, respectively, through a rubber hose attached to the base of the pipette needle. Separate syringes, needles and rubber hoses were used for each treatment to prevent cross-contamination. Larvae that bled excessively following injections were discarded. Insects were then placed on artificial diet in separate containers 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, TmEH-2

Previously in our laboratory, a full-length EH cDNA (TmEH-1; NCBI accn. U73680) was cloned from a library made with the fat body of *T. ni* (Harris et al., 1999). This cDNA library was constructed with L5D3 larvae. This 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 (Harris et al., 1999) suggested that

multiple EH genes may be present in *T. ni* larvae. Recent reports have shown the presence of multiple insect EH cDNAs in *C. felis* (2 EH cDNAs) and *D. melanogaster* (3 EH cDNAs). In the present study, a cDNA library was constructed 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 accn. 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* (Harris et al., 1999). A putative EH cDNA, TmEH-2 (NCBI accn. AF035482), was subsequently cloned and sequenced from the *T. ni* gut library (Figure 1). This cDNA contained the complete coding sequence of a protein predicted to have EH activity. TmEH-2 and the $TmEH_{(237)}$ probe were 81% identical, and the region of overlap between these two sequences (found in the translated region) is single-underlined in Figure 1. The length of the TmEH-2 insert was 2054 bp, with 5'- and 3'-untranslated regions consisting of 29 and 636 bp, respectively. The putative polyadenylation signal, ATTAAA, began at nucleotide 1966 (double underlined, Figure 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. The two EHs (TmEH-1 and -2) appear to be unique based on these variations and the percent 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). 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*. TmEH-1 and TmEH-2 are at least different alleles of the same gene, and may be 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 percent identities ranged from 42 to 47% when TmEH-2 was compared to other insect EH amino acid sequences. Interestingly, TmEH-2 was similar to microsomal EHs 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.

Nucleotide and deduced amino acid sequences of T. ni EH cDNA, TmEH-3

A portion of a third EH cDNA was isolated in our studies, TmEH-3 (NCBI accn. BG354599). The partial nucleotide sequence of TmEH-3 and the predicted amino acid sequence are shown in Figure 2. The TmEH-3 cDNA is ~ 1500 bp as determined by agarose gel electrophoresis. A total sequence of 789 bp was obtained. Conceptual translation of the sequence yields one open reading frame coding for 293 amino acids. The nucleotide sequence of Tm-EH3 was 64.9 and 65.4 % identical to TmEH-1 and TmEH-2, respectively. Also, the deduced amino acid sequence was 73.8% similar (with 63.2% identity) and 75.7 % similar (with 66.2% identity) to TmEH-1 and TmEH-2, respectively. The amino acid alignment of TmEH-3 with TmEH-1 and TmEH-2 along with the human microsomal EH (HmEH) is shown in Figure 3 (see below).

While the cDNAs of TmEH-1 and TmEH-2 contained the entire coding sequence of both EHs, the cDNA of TmEH-3 contains only a portion of the sequence. After sequence analysis and alignment with other EHs, it is clear that the clone is missing about 291 bp of coding sequence from the 5' end including the start codon ATG. It is also missing the 5' untranslated region containing some of the regulatory elements of the mRNA. This EH sequence, which is approximately 35% diverged from the two previously identified EH cDNA from *T. ni*, confirms the presence of at least three EH alleles in *T. ni*.

Catalytic triad of *T. ni* mEHs

The catalytic triad residues of the EH active site were identified and their role elucidated (Lacourciere and Armstrong, 1993; Borhan et al., 1995; Linderman et al., 1995 and Arand et al., 1996). For microsomal EHs (mEHs), a covalently bound ester intermediate is formed by attack of the nucleophilic Asp (D) on the least substituted carbon atom of the epoxide ring. The intermediate ester is then hydrolyzed by a water molecule activated by His (H) and Glu (E). The catalytic triad in the active site of the *T. ni* mEHs is denoted by asterisks in Figure 3. TmEH-2, like TmEH-1, uses the typical microsomal catalytic triad; Asp-Glu-His. Only the first amino acid of the catalytic triad is part of the sequenced region of TmEH-3. Like the other *T. ni* mEHs, TmEH-3 retains Asp acid as the first amino acid of the catalytic triad. For soluble EHs (sEHs), the catalytic triad is similar to the mEHs but the Glu residue is replaced by Asp. Recent studies on the sEHs suggest that two other tyrosine residues are involved in the formation of the ester intermediate (Argiriadi et al., 2000; Rink

```
   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  ttgtatttaatcgcagtcttgttgtgttgacaacatttactttaaatatattatgcaacctgttttttttgttattaaatt

1974    atcttacataaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
```

Figure 1. Nucleotide and deduced amino acid sequences of the full-length epoxide hydrolase cDNA (TmEH-2; NCBI accn. 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 accn. U35736) used for cDNA library screening is single-underlined in the translated region. The proposed amino acid residues of the catalytic triad are indicated by asterisks: D*, E* and H*. The putative polyadenylation signal is double-underlined.

```
  1 ttgagctactgggcagaggagtacaatttcagtgaacgagagacc
    L  S  Y  W  A  E  E  Y  N  F  S  E  R  E  T
 46 ttcctgaaccagttcccccactacaagacttacatacagggtctg
    F  L  N  Q  F  P  H  Y  K  T  Y  I  Q  G  L
 91 gatatccacttcatcagggtgaagccacaggtaccacaaaatgtg
    D  I  H  F  I  R  V  K  P  Q  V  P  Q  N  V
136 gagattgtcccacttctcttaatgcacggctggccagggtctgtg
    E  I  V  P  L  L  L  M  H  G  W  P  G  S  V
181 cgagagttctatgaagccattcctctgctcacccgccagcaacca
    R  E  F  Y  E  A  I  P  L  L  T  R  Q  Q  P
226 ggatacaactttgctttcgaagttattgtaccaagtatacctgga
    G  Y  N  F  A  F  E  V  I  V  P  S  I  P  G
271 tatggattttcacaaggagccgtccgcccggggctcggagcacct
    Y  G  F  S  Q  G  A  V  R  P  G  L  G  A  P
316 caagtatcagtgatcttcaagaacctgatgaaccggctcgggtac
    Q  V  S  V  I  F  K  N  L  M  N  R  L  G  Y
361 gacaagttctacattcagggaggagactggggcgcagtaatagcg
    D  K  F  Y  I  Q  G  G  D  W  G  A  V  I  A
406 tctactatggctactatattcccagaattgcttcttggacatcat
    S  T  M  A  T  I  F  P  E  L  L  L  G  H  H
451 tcaaacatgctgacggttcataacagcaaatcaacgttgaagatg
    S  N  M  L  T  V  H  N  S  K  S  T  L  K  M
496 ttcatcggcgcatatttcccgtcgttcgtaatgcccgagcacttg
    F  I  G  A  Y  F  P  S  F  V  M  P  E  H  L
541 gtcgacagactgtacccactgtccagtttgttcgcttacgtcatg
    V  D  R  L  Y  P  L  S  S  L  F  A  Y  V  M
586 gaagagttcggctacatgcacctgcaagccactaaacctgatact
    E  E  F  G  Y  M  H  L  Q  A  T  K  P  D  T
631 attggtatacctctaacagactccccagctggtctcctcgcatat
    I  G  I  P  L  T  D  S  P  A  G  L  L  A  Y
676 attttagagaaattctccacatggacaaagaggagccacaagttc
    I  L  E  K  F  S  T  W  T  K  R  S  H  K  F
721 aaagaagacggtggccttgaattcaggttcacaaaagaccagctc
    K  E  D  G  G  L  E  F  R  F  T  K  D  Q  L
766 ctagacaatctaatgatctactgg 789
    L  D  N  L  M  I  Y  W
```

Figure 2. Nucleotide sequence and deduced amino acid sequences of the partial epoxide hydrolase cDNA (TmEH-3; NCBI accn. BG354599).

et al., 2000; Yamada et al., 2000; and Gomez et al., 2004). Rink et al. (2000) compared the amino acid sequence of several mEHs and sEHs and showed that there are two conserved tyrosine residues (Y2 and Y3 in Figure 3) that are expected to play a role in nucleophilic addition. The tyrosine residues conserved in the three mEHs from *T. ni* are shown in Figure 3 in comparison with the human mEH. The tyrosine residues Y1, Y2 and Y3 are conserved in all mEHs (Rink et al., 2000) while Y2 and Y3 are conserved in both sEHs and mEHs. These three tyrosine residues are conserved in TmEH-2. The Y1 and Y2 residues are present and conserved in TmEH-3. Y3 is outside the sequenced region of TmEH-3.

```
      1                                                    50
TmEH1 MGRLLFLVPV LAIVLLPVYY LFLQGPPPLP DLDYNEWWGP ES.GKQKQDT
TmEH2 MARLLFILPV LALVFLPVYF LFLQSPPPVP NVDMNDWWGP ES.AKEKQDT
TmEH3 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HmEH ~~~~MWLEIL LTSVLGFAIY WFISRDKEET LPLEDGWWGP GTRSAAREDD

      51                                                   100
TmEH1 SVRPFKINFG ENLVKDLKDR LKRTRPLTPP LEGVGFEYGF NTNEINSWLK
TmEH2 SIRPFKISFG NNNVKDLKDR LQRTRPLTPP LEGVGFDYGF NTNEIDSWLK
TmEH3 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~LS
 HmEH SIRPFKVETS DEEIHDLHQR IDKFR.FTPP LEDSCFHYGF NSNYLKKVIS

      101                                                  150
TmEH1 YWAEGYNFKE RETFLNQFPQ FKTNIQGLDI HFIKVT.PKV PAGVQVVPML
TmEH2 YWAKDYNFKE RETFLNQFPQ FKTNIQGLDI HFIRVT.PKV PQGVEVVPLL
TmEH3 YWAEEYNFSE RETFLNQFPH YKTYIQGLDI HFIRVK.PQV PQNVEIVPLL
 HmEH YWRNEFDWKK QVEILNRYPH FKTKIEGLDI HFIHVKPPQL PAGHTPKPLL

      151                                                  200
TmEH1 LLHGWPGSVR EFYESIPLLT AVSK...DRD FALEVIVPSL PGYGFSDGAV
TmEH2 LLHGWPGSVR EFYEAIPLLT AVSK...DRD FAFEVIVPSL PGYGFSDPAV
TmEH3 LMHGWPGSVR EFYEAIPLLT RQQP...GYN FAFEVIVPSI PGYGFSQGAV
 HmEH MVHGWPGSFY EFYKIIPLLT DPKNHGLSDE HVFEVICPSI PGYGFSEASS

      201                                 *                250
TmEH1 RPGMGAPHIG IIMRNLMNRL GYKRYFVQGG DWGSVIGTSL ATFFPEEVLG
TmEH2 RPGLGAPQIG VVMKNLMSRL GYKQFYLQGG DWGALIGNCI VTLFPKDILG
TmEH3 RPGLGAPQVS VIFKNLMNRL GYDKFYIQGG DWGAVIASTM ATIFPELLLG
 HmEH KKGFNSVATA RIFYKLMLRL GFQEFYIQGG DWGSLICTNM AQLVPSHVKG

      251                                       Y1         300
TmEH1 YHANIGLVLS TKAMVWQAIG SVWPSLIMDD LSLVDRIYPL .SKTLSFQVR
TmEH2 YHTNMPIVMS AKSTLFELLG SVFPSLILED MSTYERLYPL .STRFANLLR
TmEH3 HHSNMLTVHN SKSTLKMFIG AYFPSFVMPE .HLVDRLYPL .SSLFAYVME
 HmEH LHLNMALVLS NFSTLTLLLG QRFGRFLGLT ERDVELLYPV KEKVFYSLMR

         Y2                                                350
TmEH1 ESGYLHIQAS KPDTVGVALT DSPAGLLAYI VEKFSIWTRP ELTSKPNGGL
TmEH2 ETGYMHIQST KPDTVGVALS DSPAGLLAYI LEKFATWTRP DLMSKPNGGL
TmEH3 EFGYMHLQAT KPDTIGIPLT DSPAGLLAYI LEKFSTWTKR SHKFKEDGGL
 HmEH ESGYMHIQCT KPDTVGSALN DSPVGLAAYI LEKFSTWTNT EFRYLEDGGL

      351                               Y3                 400
TmEH1 DFRFTKDQLI DNLMMYWTSK SITTSVRLYA ESFNIKVLGY QLDDIPTPVP
TmEH2 DYRFTRDQLI DNLMMYWTNR AITPAMRLYA ENFNKRTVEM KLDEIPTPVP
TmEH3 EFRFTKDQLL DNLMIYW~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HmEH ERKFSLDDLL TNVMLYWTTG TIISSQRFYK ENLGQGWMTQ KHERMKVYVP

      401     *                                 *            450
TmEH1 SWFIQGKYEI AYQPPFVLKL KYPNIVGVTV LDDGGHFFAF ELPEVFSKDV
TmEH2 TWGLQTKYEL GYQPKYILKI KFPNLVGTTV LQEGGHFIAF ELPEVFTNDV
TmEH3 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HmEH TGFSAFPFEL LHTPEKWVRF KYPKLISYSY MVRGGHFAAF EEPELLAQDI

      451                469
TmEH1 LKAVTAFRKL QKNNEKTDL
TmEH2 IKAVTEFRKL QKKNVKTDL
TmEH3 ~~~~~~~~~~ ~~~~~~~~~
 HmEH RKFLSVLERQ ~~~~~~~~~
```

Figure 3. Alignment of deduced amino acid sequences for TmEH-1, TmEH-2 and TmEH-3 compared to the Human mEH (HmEH). The catalytic triad D/E/H is indicated by asterisks and the tyrosine residues (Y) conserved in all mEHs are indicated by Y1, Y2 and Y3 as explained by Rink et al. (2000).

Baculovirus expression of TmEH-1 *in vivo* in *T. ni* larvae

The involvement of JH esterase in the regulation of insect development has been clearly demonstrated in Lepidoptera by selective *in vivo* enzymatic inhibition (Roe et al., 1997) and by the appearance of developmental abnormalities in larvae infected with a JH esterase-transformed baculovirus (Hammock et al., 1990). Injection of a JH esterase baculovirus into second stadium *M. sexta* led to blackened larvae. Feeding the virus to first stadium larvae reduced weight and growth when compared to control virus treatments. Both of these conditions likely are due to an abnormal decrease in JH titer caused by an 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.

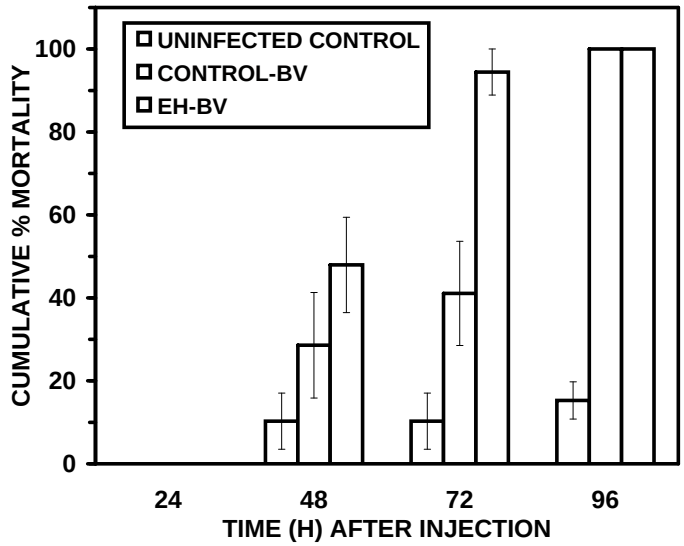

Figure 4. Effects of *in vivo* expression of TmEH-1 recombinant, non-occluded baculovirus in *T. ni* larvae. Insects were injected at L5D0 with 2 µL of complete medium containing 3.0 x 10^5 pfu of either EH-transformed baculovirus (EH-BV) or non-transformed, wild-type control baculovirus (Control-BV). 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 ± 1 SEM.

As discussed earlier, the importance of EH in decreasing JH titer and the regulation of metamorphosis is unknown. A major reason for this uncertainty has been the difficulty in developing potent *in vivo* inhibitors of insect EH and determining the effects of EH inhibition on insect development and endogenous JH levels. In an attempt to determine the importance of EH in larval development, the TmEH-1 cDNA was subcloned into a baculovirus expression system and expressed *in vivo* in *T. ni* larvae. Only TmEH-1 was used in these studies since there was a high percent identity between this EH and TmEH-2. In addition, Anspaugh (2003) showed that both EHs were expressed in fat body and gut, tissues known to be important in JH metabolism. Finally, TmEH-1 expressed in a *Spodoptera frugiperda* (Sf9) cell line was JH-specific when compared to other epoxide substrates (Harris et al., 1999). If TmEH-1 plays a role in clearing JH *in vivo*, its overexpression by the transformed baculovirus should cause a decrease in the JH titer and precocious metamorphosis. L5D0 *T. ni* larvae were treated by injection with either 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 over the course of the experiment. Furthermore, the TmEH-1 virus caused no significant reduction in body weight as compared to the control (data not shown). Surprisingly, however, the TmEH-1 baculovirus produced a higher larval mortality in a shorter time post injection as compared to the control (Figure 4). While no mortality occurred at 24 h post-injection, the TmEH-1 baculovirus elicited 48% cumulative mortality at 48 h compared to 29% for the control. The differences were 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% at any time during larval

development. The increase in the rate of mortality for TmEH-1 baculoviruses was similar to the lethal times for a scorpion peptide neurotoxin-transformed baculovirus injected into the silkworm, *Bombyx mori* (Maeda et al., 1991). In these studies, insects died between 60-75 h after injection.

The mechanism responsible for the increase in the rate of mortality post-infection for the EH-transformed baculovirus in *T. ni* is unknown. The enhanced virulence does not appear to be explained by an increase in JH metabolism, since there was no increase in the whole body JH III EH activity as compared to controls (data not shown). However, TmEH-1 baculovirus-treated larvae did have higher levels of expressed EH protein typical of that previously shown by SDS-PAGE for this same EH expressed in cell culture (Harris et al., 1999). TmEH-1 JH III epoxide hydrolase activity might have been susceptible to proteolytic digestion specifically associated with a baculovirus infection. Otherwise *T.* ni EH activity is not especially labile and is stable in crude insect homogenates and during protein purification. The viral 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). It is also possible that the mechanism of enhanced toxicity for baculovirus expressed TnEH-1 is non-specific. Because the enzyme is hydrophobic, overexpression and incorporation of the protein into cell membranes could interfere with normal cell membrane function. 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 non-occluded baculovirus killed *T. ni* larvae significantly faster (within 60 h) than the non-transformed control virus after injection. 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 but may have some significance in the application of these viruses in insect control (Possee and Bishop, 1992; Bonning and Hammock, 1996; Possee et al., 1997). Further studies are needed to better understand the mechanism for the apparent increased virulence of the EH transformed virus.

Summary

The cloning of two new EH cDNAs (TmEH-2, TmEH-3) from *T. ni* has provided further evidence for the existence of multiple EH alleles or genes in this species. Amino acid alignments suggest that TmEH-2 and TmEH-3, like TmEH-1, are microsomal enzymes. *In vivo* baculovirus expression of TmEH-1 in *T. ni* larvae did not resolve 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.

Acknowledgements

This research was supported by the North Carolina Agricultural Research Service and the National Science Foundation (IBN-9982445). Sayed Khalil was supported by a fellowship from the Institute of International Education and a research assistantship from the Department of Entomology at NC State University.

References

Anspaugh D. D. (2003). Molecular Genetics and Enzyme Regulation of Epoxide Hydrolases in the Cabbage Looper, *Trichoplusia ni*. Ph. D. Dissertation, North Carolina State University.

Arand, M., Wagner, H., Oesch, F., 1996. Asp^{333}, Asp^{495}, and His^{523} form the catalytic triad of rat soluble epoxide hydrolase. The Journal of Biological Chemistry **271** (8), 4223-4229.

Argiriadi M. A., Morisseau C., Goodrow M. H., Dowdy D. L., Hammock B. D. and Christianson D. W. (2000). Binding of alkylurea inhibitors to epoxide hydrolase implicates active site tyrosines in substrate activation. *J. Biol. Chem.* **275**, 15265-15270.

Bonning, B.C., Hammock, B.D., 1996. Development of recombinant baculoviruses for insect control. Annual Reviews in Entomology **41**, 191-210.

Borhan, B., Jones, A.D., Pinot, F., Grant, D.F., Kurth, M.J., Hammock, B.D., 1995. Mechanism of soluble epoxide hydrolase. The Journal of Biological Chemistry **270** (45), 26923-26930.

Brooks, G.T., Harrison, A., Lewis, S.E., 1970. Cyclodiene epoxide ring hydration by microsomes from mammalian liver and houseflies. Biochemical Pharmacology **19**, 225-273.

Brooks, G.T., 1974. Chlorinated Insecticides, Vol. 2. Biological and Environmental Aspects. CRC Press, Cleveland, OH.

Brooks, G.T., 1977. Epoxide hydratase as a modifier of biotransformation and biological activity. General Pharmacology **8**, 221-226.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry **162**, 156-159.

Gilbert, L.I., Granger, N.A., Roe, R.M., 2000. The juvenile hormones: historical facts and speculations on future research directions. Insect Biochemistry and Molecular Biology **30**, 617-644.

Gomez G. A., Morisseau C., Hammock B. D. and Christianson D. W. (2004). Structure of human epoxide hydrolase reveals mechanistic inferences on bifunctional catalysis in epoxide and phosphate ester hydrolysis. *Biochemistry*. **43**, 4716-23.

Guengerich, F.P., 1982. Epoxide hydrolase: properties and metabolic roles. Reviews in Biochemical Toxicology **4**, 5-30.

Guenthner, T.M., Oesch, F., 1981. Microsomal epoxide hydrolase and its role in polycyclic aromatic hydrocarbon biotransformation. In: Gelboin, H.V., Ts'o, P.O.P. (Eds.), Polycyclic Hydrocarbons and Cancer, Vol. 3. Academic Press, New York, pp.183-212.

Halarnkar, P.P., Wixtrom, R.N., Silva, M.H., Hammock, B.D., 1989. Catabolism of epoxy fatty esters by the purified epoxide hydrolase from mouse and human liver. Archives of Biochemistry and Biophysics **272** (1), 226-236.

Halarnkar, P.P., Nourooz-Zadeh, J.T., Kuwano, E., Jones, A.D., Hammock, B.D., 1992. Formation of cyclic products from the diepoxide of long-chain fatty esters by cytosolic epoxide hydrolase. Archives of Biochemistry and Biophysics. **294** (2), 586-593.

Halarnkar, P.P., Jackson, G.P., Straub, K.M., Schooley, D.A., 1993. Juvenile hormone catabolism in *Manduca sexta*: homologue selectivity of catabolism and identification of a diol-phosphate conjugate as a major end product. Experientia **49**, 988-994.

Hammock, B.D., 1985. Regulation of juvenile hormone titer: degradation. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 7. Pergamon Press, New York, pp. 431-472.

Hammock, B.D., Bonning, B.C., Possee, R.D., Hanzlik, T.N., Maeda, S., 1990. Expression and effects of the juvenile hormone esterase in a baculovirus vector. Nature **344**, 458-461.

Harris, S., Marin Thompson, D., Linderman, R.J., Tomalski, M.D., Roe, R.M., 1999. Cloning and expression of a novel juvenile hormone-metabolizing epoxide hydrolase during larval-pupal metamorphosis of the cabbage looper, *Trichoplusia ni*. Insect Molecular Biology **8** (1), 85-96.

Israel, D.I., 1993. A PCR-based method for high stringency screening of cDNA libraries. Nucleic Acids Research **21**, 2627-2631.

Kallapur, V.L., Majumder, C., Roe, R.M., 1996. *In vivo* and *in vitro*-tissue specific metabolism of juvenile hormone during the last stadium of the cabbage looper, *Trichoplusia ni*. Journal of Insect Physiology **42**, 181-190.

Keiser, K.C.L., Brandt, K.S., Silver, G.M., Wisnewski, N., 2002. Cloning, partial purification and in vivo developmental profile of expression of the juvenile hormone epoxide hydrolase of *Ctenocephalides felis.* Archives of Insect Biochemistry and Physiology **50**, 191-206.

Korth, K.L., Levings, C.S. III, 1993. Baculovirus expression of the maize mitochondrial protein URF13 confers insecticidal activity in cell cultures and larvae. Proceedings of the National Academy of Sciences USA **90**, 3388-3392.

Lacourciere, G.M., Armstrong, R.N., 1994. Microsomal and soluble epoxide hydrolases are members of the same family of C-X bond hydrolase enzymes. Chemical Research in Toxicology **7** (2), 121-124.

Lacourciere, G.M., Armstrong, R.N., 1993. The catalytic mechanism of microsomal epoxide hydrolase involves an ester intermediate. Journal of the American Chemical Society **115**, 10466-10467.

Lake, B.G., Kozlen, S.L., Evans, J.G., Gray, T.J.B., Young, P.J., Gangolli, S.D., 1987. Effect of prolonged administration of clofibric acid and di-(2-ethylhexyl)phthalate on hepatic enzyme activities and lipid peroxidation in the rat. Toxicology **44**, 213-228.

Lewin, B. 1994. Genes V. Oxford University Press, New York.

Linderman, R.J., Walker, E.A., Haney, C., Roe, R.M., 1995. Determination of the regiochemistry of insect epoxide hydrolase catalyzed epoxide hydration of juvenile hormone by ^{18}O-labeling studies. Tetrahedron **51**, 10845-10856.

Maeda, S., Volrath, S.L., Hanzlik, T.N., Harper, S.A., Maddox, D.W., Hammock, B.D., Fowler, E., 1991. Insecticidal effects of an insect-specific neurotoxin expressed by a recombinant baculovirus. Virology **184**, 777-780.

Meijer, J., DePierre, J.W., 1987. Hepatic levels of cytosolic, microsomal and mitochondrial epoxide hydrolases and other drug-metabolizing enzymes after treatment of mice with various xenobiotics and endogenous compounds. Chemico-Biological Interactions **62**, 249-269.

Meijer, J., DePierre, J.W., 1988. Cytosolic epoxide hydrolase. Chemico-Biological Interactions **64**, 207-249.

Mullin, C.A., 1988. Adaptive relationships of epoxide hydrolase in herbivorous arthropods. Journal of Chemical Ecology **14** (10), 1867-1888.

Nourooz-Zadeh, J.T., Uematsu, B., Borhan, B., Kurth, M.J., Hammock, B.D., 1992. Characterization of the cytosolic epoxide hydrolase-catalyzed hydration products from 9,10:12,13-diepoxy stearic esters. Archives of Biochemistry and Biophysics **294**, 675-685.

O'Reilly, D.R., Miller, L.K., Luckow, V.A., 1992. Baculovirus Expression Vectors: a Laboratory Manual. W.H. Freeman, New York.

Oesch, F., 1973. Mammalian epoxide hydrolases: inducible enzymes catalysing the inactivation of carcinogenic and cytotoxic metabolites derived from aromatic and olefinic compounds. Xenobiotica **3**, 305-340.

Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., Silman, I., Schrag, J., Sussman, J.S., Verschueren, K.H.G., Goldman, A., 1992. The / hydrolase fold. Protein Engineering **5**, 197-211.

Possee, R.D., Bishop, D.H.L., 1992. Baculoviruses as expression vectors and genetically engineered insecticides. In: Murray, J.A.H. (Ed.), Transgenesis. John Wiley and Sons, New York, pp. 105-129.

Possee, R.D., Barnett, A.L., Hawtin, R.E., King, L.A., 1997. Engineered baculoviruses for pest control. Pesticide Science **51**, 462-470.

Rink R., Kingma J., Spelberg J. H. L. and Janssen D. B. (2000). Tyrosine Residues Serve as Proton Donor in the Catalytic Mechanism of Epoxide Hydrolase from *Agrobacterium radiobacter*. *Biochemistry* **39**, 5600 – 5613.

Roe, R.M., Venkatesh, K., 1990. Metabolism of juvenile hormones: degradation and titer regulation. In: Gupta, A.P. (Ed.), Morphogenetic Hormones of Arthropods, Vol. 1. Rutgers University Press, New Brunswick, N.J., pp. 126-179.

Roe, R.M., Hammond, A.M. Jr., Sparks, T.C., 1982. Growth of larval *Diatraea saccharalis* (Lepidoptera: Pyralidae) on artificial diet and synchronization of the last larval stadium. Annals of the Entomological Society of America **75**, 421-429.

Roe, R.M., Anspaugh, D.D., Venkatesh, K., Linderman, R.J., Graves, D.M., 1997. A novel geminal diol as a highly specific and stable *in vivo* inhibitor of insect juvenile hormone esterase. Archives of Insect Biochemistry and Physiology **36**, 165-179.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: a Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Seidegard, J., DePierre, J.W., 1983. Microsomal epoxide hydrolase. Properties, regulation and function. Biochimica et Biophysica Acta **695**, 251-270.

Slade, M., Brooks, G.T., Hetnarski, H.K., Wilkinson, C.F., 1975. Inhibition of the enzymatic hydration of the epoxide HEOM in insects. Pesticide Biochemistry and Physiology **5**, 35-46.

Taniai K., Inceoglu A. B., Yukuhiro K. and Hammock B. D. (2003). Characterization and cDNA cloning of a clofibrate-inducible microsomal epoxide hydrolase in *Drosophila melanogaster*. *Eur. J. Biochem.* **270**, 4696-705.

Wixtrom, R.N., Hammock, B.D., 1985. Membrane-bound and soluble-fraction epoxide hydrolases. In: Zakim, D., Vessey, D.A. (Eds.), Biochemical Pharmacology and Toxicology, Vol 1: Methodological Aspects of Drug Metabolizing Enzymes. John Wiley and Sons, New York, pp. 1-93.

Wojtasek, H.W., Prestwich, G.D., 1996. An insect juvenile hormone-specific epoxide hydrolase is related to vertebrate microsomal epoxide hydrolases. Biochemical and Biophysical Research Communications **220**, 323-329.

Yamada T., Morisseau C., Maxwell J. E., Argiriadi M. A., Christianson D. W. and Hammock B. D. (2000). Biochemical evidence for the involvement of tyrosine in epoxide activation during the catalytic cycle of epoxide hydrolase. *J. Biol. Chem.* **275**, 23082-23088