Abstract

KHALIL, SAYED MOHAMED SAYED. Physiological and Molecular Studies of the Digestive System and the Juvenile Hormone Metabolizing Enzymes in Selected Lepidoptera (under the direction of Dr. R. Michael Roe)

Following the success using *Bacillus thuringiensis* protein toxins for pest control, there is great interest in finding new pesticide targets in the insect gut. To investigate possible molecular targets in the digestive system of the cabbage looper, *Trichoplusia ni*, we sequenced 49 expressed sequence tags (ESTs) from a cDNA library made from the digestive system of the last stadium of day 1 and day 2 larvae. Among these ESTs, 44 were high quality sequences and were subjected to amino acid and nucleic acid alignments with sequences in the GenBank database. Thirty percent of the ESTs were novel, 7% matched with sequences of unknown functions and 63% matched with sequences of known functions. As might be expected, one of the most abundant classes of ESTs (8/44 ESTs, 18%) codes for digestive enzymes. Another 18% of the ESTs code for elements required for nucleic acid or protein synthesis. The rest of the ESTs code for signal transduction molecules, other cellular structures, and enzymatic activities. A new epoxide hydrolase gene (EH) that is different from TmEH-1 and TmEH-2 previously isolated from *T. ni* larvae was identified. The current study is part of a larger *T. ni* EST project (~1000 ESTs) available on GenBank.

The role of juvenile hormone esterase (JHE) and EH in reproduction of the cotton bollworm, *Helicoverpa zea*, was investigated. Peak emergence of male and female

bollworm adults occurred early in the scotophase. Female adults were mixed with males in a 1:2 ratio, respectively, at the beginning of the first photophase after emergence. The highest oviposition rates for mated females were noted on d 2-4 post-emergence. The *in vitro* JH III esterase and JH III EH activity was measured in whole body homogenates of virgin and mated females from d 1 to d 8 post-emergence. Maximal JHE activity for virgin females occurred on d 2 which was approximately twice that of mated females on the same day. The same results were observed for EH. By d 4, both JHE and JH EH activities declined significantly in virgin and mated females and were the same between sexes through d 7. The developmental changes and effects of mating on JH metabolic activity were similar when measured per mg protein and per insect. The highest levels of JHE and JH EH activity in d 2 virgin and mated females was found in ovaries followed by the carcass and then hemolymph; no EH activity was found in hemolymph. For ovary, the JHE and JH EH activity was highest in virgin compared to mated females. The role of both enzymes in the regulation of reproduction is discussed.

In insects, EHs are believed to play a role in xenobiotic transformation and JH metabolism. The Roe lab at North Carolina State University isolated two EH cDNAs, TmEH-1 and TmEH-2, from the fat body and the digestive system of the fifth stadium of *T. ni* larvae, respectively. To study the difference between these two EHs and their functional role in *T. ni,* an attempt to express the full-length cDNAs was conducted using the InsectSelect^{TM} Glow system. In separate experiment, only the ORF of both EHs was used. PCR of genomic DNA from transformed cells, resistance to the antibiotic Zeocin, and GFP expression indicated the incorporation of the expression vector into the cell

genome. Measurement of EH activity and SDS-PAGE analysis showed that the EH genes were not expressed in the transformed cells.

Physiological and Molecular Studies of the Digestive System and the Juvenile Hormone Metabolizing Enzymes in Selected Lepidoptera

by

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Dedication

I dedicate all the work and effort that has gone into this project to my parents whose support and encouragement have made me who I am today. I also want to say thank you to my wife, who listened, helped, supported and inspired me during this work.

Biography

Sayed Khalil was born in Cairo, Egypt in 1967. He received a Bachelor of Science degree in Biochemistry and Chemistry from Ain Shams University, Cairo, Egypt, in 1989. In 1991, he joined the National Institute of Oceanography and Fisheries (NIOF) and in 1993 he moved to the Agricultural Genetic Engineering Research Institute (AGERI), Ministry of Agriculture and Land Reclamation, Egypt. During his employment, he got his Master of Science degree in Biochemistry from Ain Shams University in 1998. In 1999, he joined North Carolina State University to study his Ph. D. In 2004, he got his Ph. D. in Entomology and Biotechnology under the direction of Dr. R. Michael Roe, Department of Entomology at NC State.

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CHAPTER 1

Expressed Sequence Tags (ESTs) and a Partial Sequence of a New Epoxide Hydrolase from the Digestive System of the Cabbage Looper, *Trichoplusia ni*

Abstract

Following the success using *Bacillus thuringiensis* protein toxins for pest control, there is great interest in finding new pesticide targets in the insect gut. To investigate possible molecular targets in the digestive system of the cabbage looper, *Trichoplusia ni*, we sequenced 49 expressed sequence tags (ESTs) from a cDNA library made from the digestive system of the last stadium of day 1 and day 2 larvae. Among these ESTs, 44 were high quality sequences and were subjected to deduced amino acid and nucleic acid alignments with sequences in the GenBank database. Thirty percent of the ESTs were novel, 7% matched with sequences of unknown functions and 63% matched with sequences of known functions. As might be expected, one of the most abundant classes of ESTs (8/44 ESTs, 18%) coded for digestive enzymes, i. e., 5 for trypsin, 1 for chymotrypsin, 1 for diverged serine protease and 1 for lipase. Alignment of the 5 trypsin ESTs indicated 3 different types of trypsin activity are expressed in the *T. ni* midgut. Another 18% of the ESTs code for elements required for nucleic acid or protein synthesis. The rest of the ESTs code for signal transduction molecules, other cellular structures and enzymatic activities. A new epoxide hydrolase gene (EH) that is different from TmEH-1 and TmEH-2 previously isolated from *T. ni* larvae was identified. The nucleic acid sequence of the new EH was 64.9 and 65.4 % identical to TmEH-1 and TmEH-2, respectively. Also, the deduced amino acid sequence of the new EH was 73.8 and 75.7 % similar to TmEH-1 and TmEH-2, respectively. The current study is part of a larger *T. ni* EST project (~1000 ESTs) available on GenBank.

Introduction

The insect digestive system is important in food digestion and absorption, endocrine secretion (reviewed by Sehnal and Zitnan, 1996), ion movement and water balance (reviewed by Chapman, 1998 and Nation, 2002), and xenobiotic metabolism (Ishaaya and Casida,1980; Zhao et al., 1996 and Yang et al., 2004). For a long time the insect nervous system was the major target for chemical insecticides (Perry et al., 1998). However, insects successfully developed resistance to insecticides that target the nervous system (Perry et al., 1998). As a result, there has been a need to find alternative modes of pesticide action.

Interest in the insect digestive system as a target for new insecticide development has resulted from discoveries of the *Bacillus thuringiensis* (Bt) crystal protein toxins, digestive enzyme inhibitors, and trypsin modulating oostatic factor (TMOF). Great success in pest control was achieved using Bt as a bioinsecticide or by engineering Bt toxins into crop plants (reviewed by Estruch et al., 1996; Schnepf et al., 1998 and Chattopadhayay et al., 2004). Digestive enzyme inhibitors are used by plants as a defense mechanism against insects. These inhibitors are proteins that bind to the digestive enzyme blocking their active sites (reviewed by Felton and Gatehouse, 1996). TMOF is a small peptide isolated from the ovary of the yellow fever mosquito, *Aedes aegypti* (Borovsky et al., 1990) and which regulates trypsin synthesis in the gut. Reports on resistance to Bt (reviewed by Schnepf et al., 1998; Ferre and Van Rie, 2002 and Tabashnik et al., 2003) and digestive enzyme inhibitors (Gatehouse, et al., 1997; Lara et

al., 2000; Mazumdar-Leighton and Broadway, 2001 and Volpicella et al., 2003) as well as the poor insecticide activity of TMOF (Vanderherchen et al., 2004) have been the drive behind studies to find additional targets in the insect digestive system.

The insect digestive system is a rich environment for the identification of molecular components involved in digestion and absorption, ion movement or cell structure that could be targets for novel insecticides. Lepidopteran insects (~150,000 described species) are one of the most common and destructive agricultural pests in the world. The discovery of the genes expressed in the digestive system of the Lepidoptera will advance our knowledge about digestive enzymes (their synthesis and secretion), regulation of digestion, absorption, and digestive system structure and function and will enable us to find key elements that might play important roles in new control strategies or the enhancement of current insecticides that target the digestive system.

New molecular targets are currently identified using traditional biochemical techniques, molecular biology, genomics or proteomics. Expressed sequence tags (ESTs) provide information about the profile of gene expression in a specific tissue or life stage and can reveal changes that occur in the same tissue under different physiological conditions. ESTs can be used to better understand the function of different organs and to isolate new genes and to identify new targets. Eigenheer et al. (2003) identified new genes specific for the pine engraver beetle, *Ips pini,* midgut by comparing an EST database from the *Ips* midgut with that from the midgut of the silkworm, *Bombyx mori*. Landais et al. (2003) collected information about different classes of genes expressed in the fall armyworm, *Spodoptera frugiperda,* cultured cells by sequencing 5937 ESTs from

Sf9 cells. Similarly, Srisuparbh et al. (2003) identified the gene families involved in the biosynthesis of the royal jelly by establishing an EST database for the hypopharyngeal gland of the Indian honeybee, *Apis cerana*.

To further investigate digestive system structure and function, 44 ESTs from a cDNA library made from the digestive system of *T. ni* were sequenced and analyzed. These ESTs were the nucleus of a larger project comprising more than 1000 ESTs. A list of these ESTs can be found in the GenBank. From the current study, eight ESTs encoded digestive enzymes of which five represented three different trypsin-like enzymes. One of the ESTs sequenced was a putative epoxide hydrolase (EH). In insects, EHs play a role in xenobiotic metabolism as reported by Slade et al. (1975), Mullin (1988) and Taniai et al. (2003). Also, the role of EH in juvenile hormone (JH) metabolism was reviewed by Hammock (1985), Roe and Venkatesh (1990), and Gilbert et al. (2000). The Roe lab has been interested in the study of the molecular biology and biochemistry of EHs and their role in JH metabolism in insects. Two EHs were isolated in previous studies from *T. ni.* TmEH-1 was isolated from a cDNA library made from larval fat body (Harris et al., 1999). TmEH-2 was isolated from cDNA made from the larval digestive system (Anspaugh, 2003). A third EH in *T. ni* was obtained from our EST database.

Materials and Methods

cDNA library construction and plating

To build the EST library for the digestive system, we selected the actively feeding last stadium cabbage looper, *T. ni*. A cDNA library from the digestive system (after removal of malpighian tubules, tracheae and connective tissues) of the last stadium day1

(L5D1) and day2 (L5D2) *T. ni* larvae was constructed. An equal proportion of L5D1 and L5D2 *T. ni* guts was used to construct this library. The cDNA library was developed using Zap Express cDNA Synthesis and Gigapack II Gold Cloning kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The cDNA library was diluted 1:1000 in SM buffer [50mM Tris pH7.5, 100mM NaCl, 8mM $MgSO_4$, 0,01% gelatin] and 1µl was plated with XL-1 Blue *E. coli* cells in the presence of X-gal (Promega, Madison, WI) according to the instructions of the Zap Express cDNA Synthesis and Gigapack II Gold Cloning kit (Stratagene). Plates were inverted and incubated at 37°C overnight.

PCR

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 giving these amplification products were subjected to a 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.

Sequencing and bioinformatics

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). Chromatograms obtained were viewed in Chromas (Technelysium Pty Ltd, Southport, Queensland, Australia). The resulting DNA sequences were matched with sequences in the NCBI GenBank database (www.ncbi.nlm.nih.gov) using BLASTn (or BLASTx) to determine the putative function of each EST. The last GenBank search was conducted September 2004. The expected amino acid was obtained by using the ORF finder function in the NCBI website. Alignments of the nucleotides and deduced amino acid sequences of different clones were conducted using SeqWeb version 2 (Accelrys, Madison, WI).

Designing a new sequencing primer for SK59 (a new EH message) clone

The SK59 EST was a new EH gene. A new sequencing primer, EHSKF1 (5' TGAAGAACCTGATGA 3') corresponding to nucleotides 332-346 of the SK59 clone was designed to obtain additional sequence. The EHSKF1 primer was analyzed for hairpin and dimer formation using the OLIGO 4 program (MBI, West Cascade, CO) and was synthesized by Sigma-Genosys (Woodlands, TX). The sequencing reaction was conducted as described before and was sent to the Forest Biotechnology lab for sequencing.

Results and Discussions

The digestive system of insects that vector animal diseases has been extensively studied for the development of novel insecticides and disease control strategies. For example, most of our knowledge about regulation of digestion in insects is based on work with the mosquito, *Aedes aegypti* (reviewed by Borovsky 2003). Some insecticides target the digestive system of mosquitoes and have a potential use for insect control such as *Bacillus thuringiensis israelensis* (Federci eai al., 2003) and TMOF (Borovsky and Meola 2004). Looking for new targets in the insect gut, Wang and Granados (2001) reported different strategies for targeting the peritrophic membrane based on the study of its molecular structure and function.

ESTs are short, single-pass nucleotide sequences obtained from the ends of randomly selected cDNA clones (Adams et al., 1991). Because they represent portions of the coding sequences, ESTs are a fast tool for new gene discovery (Bourdon et al., 2002; McCarter et al., 2000) and for confirming coding regions in genomic sequences (Adams

et al., 1991). Gaines et al. (2002) successfully identified a large number of genes expressed only in the hindgut and Malpighian tubules of the cat flea, *Ctenocephalides felis*, by comparing ESTs from these two tissues with ESTs from the carcass.

In this study, 100 cDNA clones from the digestive system of last stadium *T. ni* were subjected to PCR analysis to determine the cDNA length contained in each clone. From these 100, 49 clones containing inserts larger than 1kb were selected for further analysis. After EST sequencing of these 49 clones, 3 sequences were unreadable. An additional two sequences were vector only, apparently without any insert. These were also removed from the analysis leaving 44 ESTs for further study To obtain the putative function of each cDNA, primer and phage sequences were removed and EST sequences were analyzed using the BLASTX algorithm (www.ncbi.nlm.nih.gov). If no match was obtained, then the BLASTN algorithm was used. BLASTX first translates the nucleotide sequence to the corresponding amino acid sequence in all six reading frames and compares them with protein sequences in the database. BLASTN finds similar nucleotide sequences in the nucleotide database. BLAST algorithms return the expect value (E) which indicates the confidence of the matches obtained. Alignments with the lower E value are most significant. The EST sequences, the search results obtained by the BLAST algorithm and the alignments of each EST with sequences in GenBank are shown in Figs. 6 and 7. Table 1 is a compilation of the 44 ESTs obtained. Their putative function (as determined by BLAST analysis), the organism from the top matching sequence obtained, and E-values of the most significant alignments are also shown.

Fig. 1 shows the percentage of ESTs that matched with organisms in the GenBank database. Of the ESTs that had similarities to a database sequence (31ESTs), the largest percentage (69%) matched with lepidopterans. Of these, 33% matched with noctuidae and 36% matched with other Lepidoptera. Twenty five percent of the ESTs matched with sequences from Diptera, i. e., 6% matched with sequences from the fruit fly, *Drosophila melanogaster*, 13% with the malaria mosquito, *Anopheles gambiae* and 6% matched with other dipterans. The remaining 6% (SK17 and SK54) matched with Hymenoptera and mammals. SK17 matched with the signal transduction molecule, the 14-3-3 protein, from the hymenopteran *Apis millifera* (honeybee). The SK54 clone matched with lipase from the pancreas of the dog, *Canis familiaris*.

The putative functions of our ESTs (Fig. 2) were categorized using the Gene Ontology Consortium (www.geneontology.org) as follows:

Novel genes (30%)

This category includes ESTs with no significant similarities to sequences in the GenBank according to the BLASTX and BLASTN algorithms (Altschul et al., 1997). This class of ESTs forms the largest subset of our ESTs and is of interest because these members might be specific to *T. ni*. This high percentage of novel ESTs is not unexpected. Schmid and Tautz (1997) found that more than one-third of their randomly sequenced cDNAs from a *D. melanogaster* library did not cross-hybridize with DNA from the most closely related species, *D. virilis*. Also, Rubin et al. (2000) reported that approximately 30% of the predicted proteins in every organism was not similar to other known organisms. Similar results were obtained from *Spodoptera frugiperda* Sf9 cells

(Landais et al., 2003), the nematode *Pratylenchus penetrans* (Mitreva et al., 2004) and the fungus *Aspragillus flavus* (Yu et al., 2004).

Unknown functions (7%)

These sequences matched with loci in the GenBank database but the functions of these database accessions are unknown. Therefore, sequence similarity did not allow us to assign a putative function in these cases. These ESTs matched with sequences obtained from *D. melanogaster* and *A. gambiae*. The matched sequences in the GenBank database resulted from the sequence of the full genome of *Drosophila* by the Berkeley *Drosophila* Genome Project, USA and from *A. gambiae* by the *Anopheles* Genome Sequencing Consortium in the European Bioinformatics Institute, United Kingdom. The novel genes and unknown functions comprised the largest portion (37%) of the ESTs, indicating the need for more research to study the functions of these genes.

Digestive enzymes (18%)

As might be expected in the digestive system of an actively feeding larva, digestive enzymes comprised a large percentage of the known putative functions identified. Trypsin, the main digestive enzyme, was represented by 5 ESTs coding for 3 different trypsin-like activities. These are represented by SK35, SK70 and SK79 (Table 1). The two other ESTs coding for trypsin (SK37 and SK72) are shorter versions of SK79. Fig. 3 shows the amino acid alignment of the three different trypsins with trypsin from *Helicoverpa armigera* isolated by Bown et al. (1997). The amino acid designated with "X" indicates that the amino acid residue is unknown because the nucleotide sequence contained an unknown nucleotide (N). Because these ESTs are single-pass sequences,

unknown nucleotides are not verified by another round of sequencing. Amino acid alignment of SK clones coding for trypsins showed a high degree of similarity between them and trypsins from other organisms. As shown in Fig. 3, SK70 and SK79 share the isoleucine (I) amino acid (indicated by an arrow) at the amino-terminus of the mature enzyme. Plus signs (+) represent the catalytic triad of trypsin enzymes. Although the first amino acid of the catalytic triad (Histidine, H_{37}) is replaced in SK70 and SK79 (SK53 is a short cDNA missing H_{37}), the second amino acid (Aspartate, D_{102}) is conserved in SK79 and SK53. The third amino acid (Serine, S_{195}) is conserved in SK53. We can not judge D_{102} in SK70 or S_{195} in SK79 and SK70 because of the unknown amino acid or a lack of additional sequence data for these two clones. The binding pocket residues (Aspartate, D_{189}; Glycine, G_{216} and Glycine, G_{226}) are conserved in SK53 (represented by the asterisks).

Finding three different trypsin cDNAs is consistent with results from other Lepidoptera. For instance, Peterson et al. (1994) identified 3 cDNAs coding for 3 different trypsins in the midgut of the tobacco hornworm, *Manduca sexta*. On the other hand, Bown et al. (1997) and Hegedus et al. (2003) identified 8 different trypsin cDNAs from the midgut of *H. armigera* and the bertha armyworm, *Mamestra configurata*, respectively. Chymotrypsin (SK16), diverged serine protease (SK28) and lipase (SK54) were also represented each by one EST. All ESTs of digestive enzymes matched with sequences from Lepidoptera except SK54 which aligned most closely with the lipase from the dog pancreas.

Nucleic acid and protein synthesis (18%)

This group was as abundant as the ESTs for digestive enzymes, reflecting the expected high synthetic activity of insect gut cells. High synthetic activity might be expected in feeding stage larvae, including the synthesis of digestive enzymes and peritrophic membrane as well as xenobiotic metabolism of secondary plant compounds. Brush border repair, intermediary metabolism and ion uptake also require protein synthesis. The *T. ni* ESTs associated with nucleic acids and protein synthesis were similar to previously identified Lepidoptera cDNAs and genes found in GenBank

Signal transduction (11%)

Signal transduction components comprise 11% of the ESTs sequenced from *T. ni* reflecting a high degree of intracellular and cell to cell communication in the insect digestive system. Calmodulin and calreticulin, two calcium binding proteins were represented by SK20 and SK22, respectively. Members of the cyclophilin and the tetraspanin families were also sequenced (SK78 and SK94, respectively). SK17 is a member of the 14-3-3 family which participates in protein kinase signaling pathways within all eukaryotic cells. Calmodulin (SK20) is a cytoplasmic protein that plays a main role in cell to cell communication, while calreticulin (SK22) is a protein that resides in the endoplasmic reticulum and is important in intra-cellular processes. Cyclophilin 1 (SK78) and Tetraspanin D76 (SK94) are members of membrane protein families with diverse functions including cell adhesion and signal transduction.

Other structural components and catalytic activities (16%)

Some ESTs coding for other cellular structural components were obtained such as SK17 coding for the family of protein related to heat shock protein (HSP), SK50 coding for actin, SK80 coding for HSP60, SK65 coding for the trachealess gene and SK76 coding for the microsatellite Ham4 (table 1). Also catalytic activities were represented by two ESTs coding for aminolevulinate synthase (SK01) and epoxide hydrolase (SK59).

SK59, a putative novel epoxide hydrolase (TmEH-3)

EHs in insects are believed to play a role in JH and xenobiotic metabolism. Two EHs were previously isolated by our lab in two different studies. The first EH, TmEH-1 (NCBI accession number U73680) was isolated by Harris et al. (1999) from a cDNA made from the fat body of the last stadium day 3 (L5D3) *T. ni*. The second EH, TmEH-2 (NCBI accession number AF035482) was isolated by Anspaugh (2003) from the same cDNA library used in this study. A portion of a new EH (SK59; TmEH-3, accession # BG354599) was isolated in this study. The partial nucleotide sequence of SK59 and the expected amino acid sequence is shown in Fig. 4. The SK59 cDNA is ~ 1500 bp as determined by agarose gel electrophoresis. Using T3 and EHSKF1 as sequencing primers, 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 (with 63.2 identity) and 75.7 % (with 66.2% identity) similar to TmEH-1 and TmEH-2, respectively. The amino acid alignment of

SK59 with TmEH-1 and TmEH-2 along with the human microsomal EH (HmEH) is shown in Fig. 5.

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 SK59 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 loci in *T. ni*.

The catalytic triad residues of the EH active site were identified and their role elucidated (Lacourciere and Armostrong, 1993; Borhan et al., 1995; Linderman et al., 1995; Arand et al., 1996 and Roe 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 Fig. 5. Only the first amino acid of the catalytic triad is part of the sequenced region of SK59. 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 suggested that two other tyrosine residues are involved in the formation of the ester intermediate (Argiriadi et al., 2000; Rink 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 Fig. 5) that are expected to play a role in nucleophilic addition. The tyrosine residues conserved in the three mEHs from *T. ni* are shown in Fig. 5 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. The Y1 and Y2 residues are present and conserved in SK59. Y3 is outside the sequenced region of SK59.

Summary

In this study we have selected 100 clones from a cDNA library made from equal portions of the digestive system of last stadium day1 (L5D1) and day2 (L5D2) *T. ni*. These clones were subjected to PCR to determine the length of the cDNA insert in each clone. Clones that contain inserts larger than 1kb were subjected to single-pass unidirectional sequencing using the universal T3 primer. Out of 49 clones, 44 were high quality sequences, 3 were unreadable, and 2 apparently had no insert. The putative functions of these ESTs were determined by alignment of the obtained sequences with sequences in the GenBank database using BLASTx and/or BLASTn. A large portion (30%) of these ESTs were novel sequences which had no significant similarity to any sequences in GenBank. Seven percent of the ESTs matched with sequences of unknown function. The rest of the ESTs showed similarities with different cellular components. As expected, digestive enzymes comprised a large group (18%) of the ESTs. Trypsin activity was the largest among this category. Trypsin was represented by 5 ESTs coding for 3 different trypsin-like activities. On the other hand, chymotrypsin, serine protease and

lipase were each represented by only one EST. Nucleic acid and protein synthesis elements constitute another large group, 18% of the ESTs, reflecting the high level of synthesis in gut cells. This is expected in feeding stage larvae because of the synthesis of digestive enzymes and peritrophic membrane as well as cell replacement. Signal transduction components constitute 11% of the ESTs including two calcium binding proteins, calmodulin and calreticulin, and three non-calcium dependent proteins, 14-3-3, cyclophilin1 and tetraspanin D76. These proteins play a major role in different metabolic pathways as part of signal transduction pathways. SK59 is a partial sequence of a new epoxide hydrolase (EH) gene. This EH is different from the two EH cDNAs isolated previously by our lab (Harris et al., 1999; Anspaugh, 2003). EHs play a role in general xenobiotic metabolism, synthetic pathways and juvenile hormone metabolism in insects. For this clone, a sequence of 789 bp coding for 293 amino acids in one open reading frame was obtained. The nucleotide sequence of the new EH was 64.9 and 65.4 % identical to TmEH-1 and TmEH-2, respectively. The deduced amino acid sequence was 73.8 (with 63.2 identity) and 75.7 % (with 66.2% identity) similar to TmEH-1 and TmEH-2, respectively.

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Table 1.1. ESTs from the digestive system of fifth stadium *Trichoplusia ni* are shown along with the GenBank accession # of each clone, the putative function, the closest matching organism, the blast algorithm used and the E-value. Dip, Diptera; Hym, Hymenoptera; Lep, Lepidoptera; NS: Not Submitted to GenBank. *A, Anopheles; C, Choristoneura; D, Drosophila; H, Helicoverpa.*

Clone	Accession #	Putative function	Organism	BLAST	E-value
No significant similarity					
SK05	NS	No significant similarity	-	blastx,n	-
SK12	NS	No significant similarity	-	blastx,n	-
SK15	BG354589	No significant similarity	-	blastx,n	-
SK21	NS	No significant similarity	-	blastx,n	-
SK29	NS	No significant similarity	-	blastx,n	-
SK30	NS	No significant similarity	-	blastx,n	-
SK35	BG354600	No significant similarity	-	blastx,n	-
SK39	NS	No significant similarity	-	blastx,n	-
SK45	NS	No significant similarity	-	blastx,n	-
SK60	NS	No significant similarity	-	blastx,n	-
SK61	CF258263	No significant similarity	-	blastx,n	-
SK73	NS	No significant similarity	-	blastx,n	-
SK82	NS	No significant similarity	-	blastx,n	-
Unknown function					
SK41	BG354601	ENSANGP00000013724	*A. gambiae* (Dip)	blastx	9e-37
SK46	BG354595	CG11139-PA	*D. melanogaster* (Dip)	blastx	3e-09
SK63	BG354603	CG33154-PA	*D. melanogaster* (Dip)	blastx	1e-13
Digestive enzymes					
SK16	NS	Chymotrypsin-like protease	*H. armigera* (Lep)	blastx	8e-32
SK28	NS	Diverged serine protease	*H. armigera* (Lep)	blastx	2e-34
SK37	BG354594	Trypsin-like protease	*H. armigera* (Lep)	blastx	2e-25
SK53	NS	Trypsin-like protease	*H. armigera* (Lep))	blastx	3e-15
SK54	BG354597	Triacylglycerol lipase	*Canis familiaris* (mammal)	blastx	1e-07
SK70	BG354604	Trypsin	*C. fumiferana* (Lep)	blastx	8e-18
SK72	CF258201	Trypsin-like protease	*H. armigera* (Lep)	blastx	1e-13
SK79	NS	Trypsin-like protease	*H. armigera* (Lep)	blastx	2e-29
Nucleic acids and protein synthesis					
SK11	NS	Reverse transcriptase	*Papilio xanthus* (Lep)	blastx	7e-12
SK38	BG354591	Y-box protein	*Bombyx mori* (Lep)	blastx	2e-34
SK49	BG354592	Elongation factor I-alpha	*Schizura sp* (Lep)	blastx	1e-41
SK57	BG354602	60S Ribosomal protein	*Bombyx mori* (Lep)	blastx	4e-99
SK58	BG354598	60S Ribosomal protein	*Bombyx mori* (Lep)	blastx	2e-53
SK80	BG354606	Elongation factor I-alpha	*Attacus atlas* (Lep)	blastx	3e-71
SK81	NS	Elongation factor I-alpha	*Attacus atlas* (Lep)	blastx	3e-63
SK83	NS	18S Ribosomal RNA	*Hyles lineata* (Lep)	blastn	1e-161
Signal transduction					
SK17	BG354593	14-3-3 protein	*Apis mellifera* (Hym)	blastx	9e-36
SK20	CF258173	Calmodulin	*A. gambiae* (Dip)	blastx	8e-34
SK22	CF259462	Calreticulin	*Bombyx mori* (Lep)	blastx	5e-60
SK78	NS	Cyclophilin 1	*D. subobscura* (Dip)	blastx	6e-39
SK49	CF259468	Tetraspanin D76	*Manduca sexta* (Lep)	blastx	2e-46

(continued next page)

Table 1.1. Continued.

Clone	Accession #	Putative function	Organism	BLAST	E-value
Cellular structures and catalytic activities					
SK19	BG354590	HSP90 related protein	*A. gambiae* (Dip)	blastx	9e-21
SK50	BG354596	Actin	*Heliothis virescence* (Lep)	blastx	6e-73
SK86	BG354607	HSP 60	*Culicoides variipennis* (Dip)	blastx	3e-78
SK65	CF258287	Bm trachaeless gene	*Bombyx mori* (Lep)	blastn	4e-07
SK76	BG354605	Microstellite Ham4	*H. armigera* (Lep)	blastn	2e-15
SK01	BG354588	Aminolevulinate synthase	*A. gambiae* (Dip)	blastx	2e-49
SK59	BG354599	Epoxide hydrolase	*Trichoplusia ni* (Lep)	blastx	7e-98

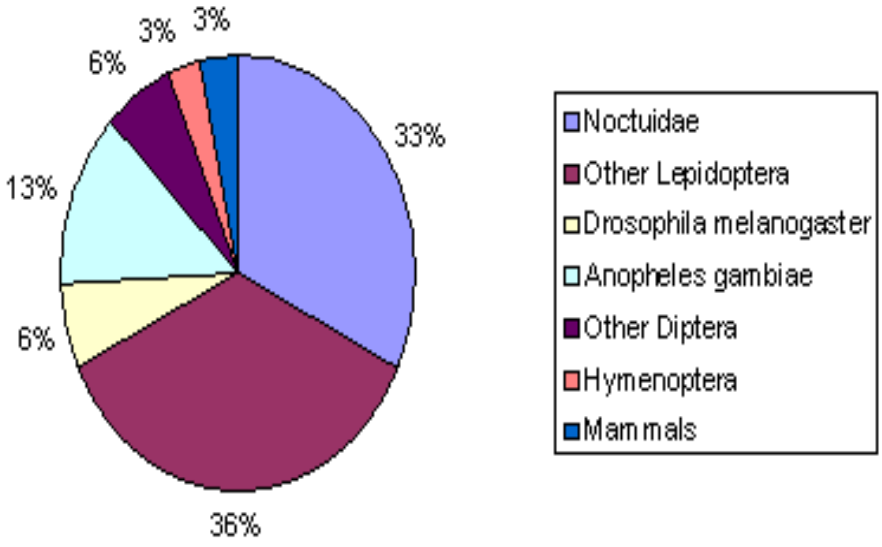

Fig. 1.1. Percentage of ESTs matching with sequences from different organisms in the GenBank database.

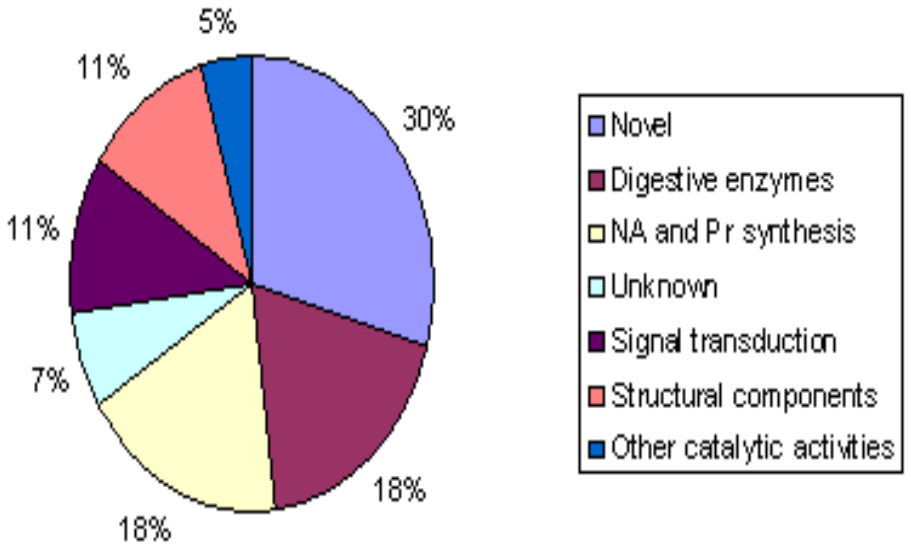

Fig. 1.2. Percentage of total ESTs with the indicated putative functions after alignments with sequences in the GenBank database. Novel means sequences that did not match with any sequences in the database, unknowns matched with sequences of unknown function. NA, nucleic acid; and Pr, protein

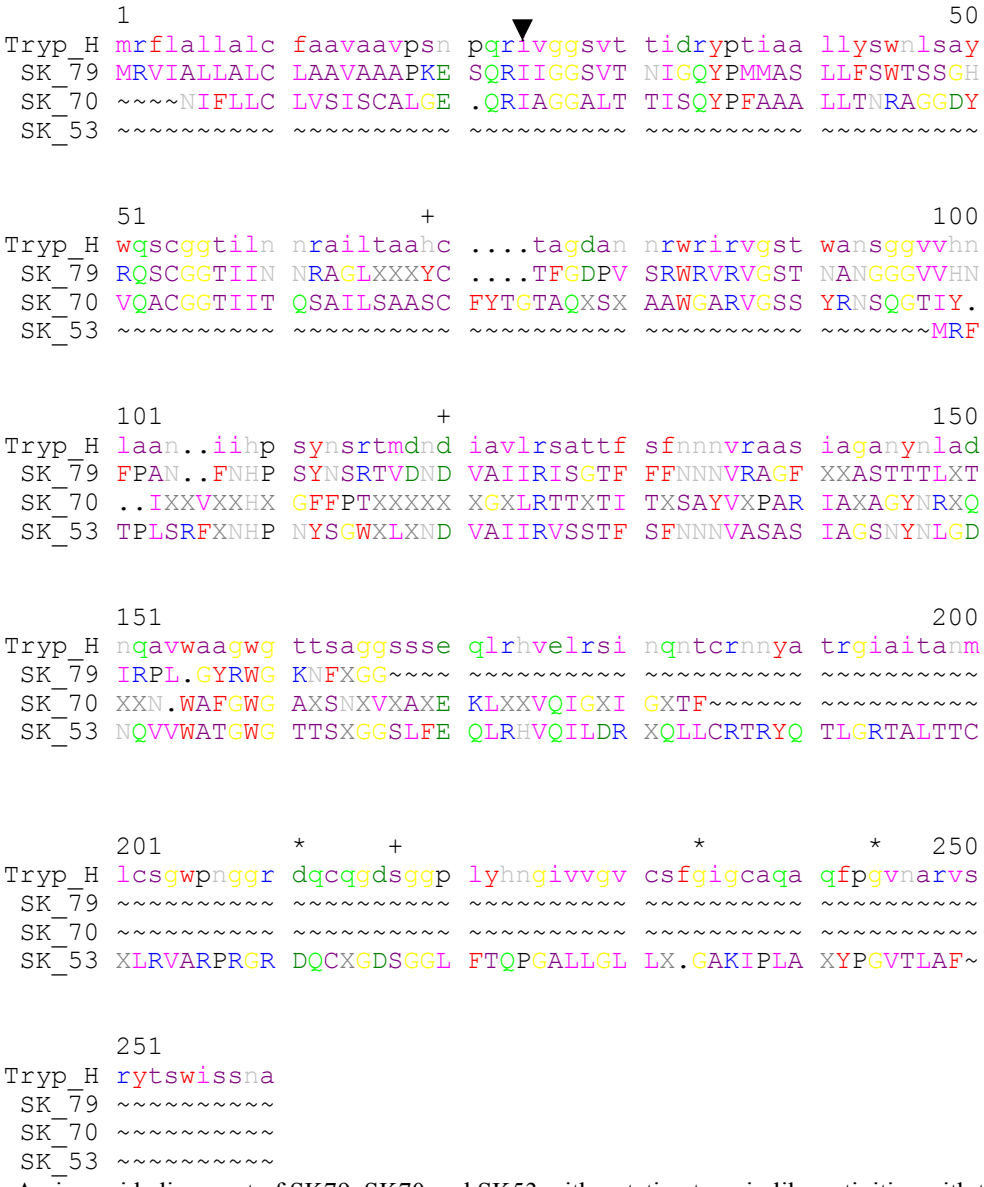

Fig. 1.3. Amino acid alignment of SK79, SK70 and SK53 with putative trypsin like activities with the amino acid sequence of the *Helicoverpa armigera* trypsin (Tryp_H) obtained from the GenBank database (accession # CAA 72962). The arrow head indicates the first amino acid (I, Isoleucine) in the mature enzyme. Plus signs (+) indicate the catalytic triad residues and the asterisks (*) indicate the binding pocket residues. Alignment was done using the pileup function of SeqWeb website 2.

```
  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
```

Fig. 1.4. Nucleotide sequence and deduced amino acid sequence of the SK59 clone which is a part of a new EH cDNA (TmEH-3). Translation obtained from the ORF function in the NCBI website. The position of the sequencing primer EHSKF1 is indicated by the green shadow.

```
      1                                                  50
TmEH1 MGRLLFLVPV LAIVLLPVYY LFLQGPPPLP DLDYNEWWGP ES.GKQKQDT
TmEH2 MARLLFILPV LALVFLPVYF LFLQSPPPVP NVDMNDWWGP ES.AKEKQDT
 SK59 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HmEH ~~~~MWLEIL LTSVLGFAIY WFISRDKEET LPLEDGWWGP GTRSAAREDD

      51                                                 100
TmEH1 SVRPFKINFG ENLVKDLKDR LKRTRPLTPP LEGVGFEYGF NTNEINSWLK
TmEH2 SIRPFKISFG NNNVKDLKDR LQRTRPLTPP LEGVGFDYGF NTNEIDSWLK
 SK59 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~LS
 HmEH SIRPFKVETS DEEIHDLHQR IDKFR.FTPP LEDSCFHYGF NSNYLKKVIS

      101                                                150
TmEH1 YWAEGYNFKE RETFLNQFPQ FKTNIQGLDI HFIKVT.PKV PAGVQVVPML
TmEH2 YWAKDYNFKE RETFLNQFPQ FKTNIQGLDI HFIRVT.PKV PQGVEVVPLL
 SK59 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
 SK59 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
 SK59 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
 SK59 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
 SK59 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
 SK59 EFRFTKDQLL DNLMIYW~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HmEH ERKFSLDDLL TNVMLYWTTG TIISSQRFYK ENLGQGWMTQ KHERMKVYVP

      401     *                             *            450
TmEH1 SWFIQGKYEI AYQPPFVLKL KYPNIVGVTV LDDGGHFFAF ELPEVFSKDV
TmEH2 TWGLQTKYEL GYQPKYILKI KFPNLVGTTV LQEGGHFIAF ELPEVFTNDV
 SK59 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HmEH TGFSAFPFEL LHTPEKWVRF KYPKLISYSY MVRGGHFAAF EEPELLAQDI

      451                469
TmEH1 LKAVTAFRKL QKNNEKTDL
TmEH2 IKAVTEFRKL QKKNVKTDL
 SK59 ~~~~~~~~~~ ~~~~~~~~~
 HmEH RKFLSVLERQ ~~~~~~~~~
```

Fig. 1.5. Alignment of deduced amino acid sequences for TmEH-1, TmEH-2 and SK59 clone 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). Alignments were conducted using the pileup function of SeqWeb 2.

Fig. 1.6. Nucleotide sequence of ESTs that showed no significant similarity to sequences in the GenBank database when analyzed using BLASTX and BLASTN algorithms.

SK05

GTTGGAGGAAACTCCGCCACTGTTGCCCCTGAAGTCGTCCNTGCGGTTCCTGCCGACTNTGTAAAACCTGAA
TCATCGCTTCTTGATATCGGTGCCAGNGTTTCGTTNCCTATCTTAGGCGACAGNTCGTTGCTNGATGTTAAT
GCTTCTCTGGGTAATATATNANATGTGGGAGCCTCGGTTGGAGGAAACTCCGCCACTGTTGCCCCTGAAGTC
GTCCNTGCGGTTCCTGCCGACTCTGTAAAACCTGAATCATCGNTTNTTGATNTCGGNGCCAGNGTTTCGTTA
CCTATNTTAGGCGACAACTCGTTGCTTGATGTTAATGCTTCTCTGGGTAATATATTAGACGTGGGAGCCTCT
GTTGGAGGAAACTCCGCCACTGTTGCCCCTGAAGTCGTCCCTGCGGTTCCTGCCGACTCTGTA

SK12

ANGANGGTTNGTCATTACTTCTTTTTTTTATTATTATTAAAGNGCAAATGACACGCACACACCCAGCGGGNG
GTAGGACATTACTTGATCGAATGTCAGCTGGNGAGGGGTTCGCTNGCTGCCTGGTGAAACTACTAGTATTTT
TCATTGAAAAAATCATTGCGAAAAACANGGACACCACATANTATATGANTGGCCTNATNCAATGAGACTCTG
TGTCCATTTCATCACTTACATNGATGCCAATTTCTACTCTAGTTAAACAATTTAGCANCGAACATTTTAANG
CCTATACTCTACATTAAATTTTCAATTAAACATATTTTTAGCACAAGTAAATATTTAGTTNCAAAGTAAGTA
GACTTCATTAAAATTTTGTTTATACCCAACCTTAATTAAAAATTAAATCGACTTGCCTATACGTGTCCAATG
TTCTACTCAAATACCTACAACATGTCTGAACAGACCGCTTTCATATTTTTAGTACCCCAGTTCAGCTCCNAA
AGGNACAAGGTTCTGCAGGAAAACCCATCATTCTGAAGAAAGCANTNGTGGNGGAAACAAAATGTTTAACGA
ANAACCAAACGNTTTTAAAATTTGGGAGCCCTAANGCTACCTGGTAAAGTGGATGCAGGGGGGGGCTTTGAN
CNCCCGGCTCTTCAACCCCTGGAAAATTTAATTNTTTGCAAAGATGGCCTTCTGCCCCNCGGGGTTTTTTTA
CCAAGNAGACCTTTNNNGG

SK15

ATCAAATATTATTTTATAATGACTTGTGCCAGGAGTGGTTCCACTTAGAGTAATGTAACTATAATGTTATTA
ACTCGGTCAGTCACATTATCTAAAAATATGAAGTGTGAATGTCAATAAGTTTATGTGATGGCTAATGTTTGA
CAGGTTAACATTTTCTATAGGTCACAGGGGCTTTTTTACCTGCTCATATTGGGCTGGTCAAATGGAAAGACT
GGGGTCTTTTGTAACCCTAAAGAAAGCCCCTTATCCCCTGCTAATATTATAAAATGGCGAAAAGGAACCTCT
GGCCTGGCCTGGCCTGGTCTGGTTACCGCTTTTCACGTCTAAAACCCCCTGGACCCGATTTACAGAAGATAG
GAGTTAACCCTTGAGAAAGAACATAGGATAGTTTTTTATCCCCGGACTTTTTGAAGAGGGTCTCTTGGAAAC
CGCGATNTANCCGAGAAAAGCTAGTATTATATATTGGTAAACAATAGCCATACATAACCAAACAGGGGGGAA
ACATAGANGNGCACCAAAATTTTTTAAANGTTGGCTCANATGAAAAAAGGTTCTTTTACCATTTGGCNAAAT
TCGAGGTTGGGAAAATTAACCTAATGGCTTTTGTTNTTANGGAAAAGGCAGNCCTGGTNAANCCAAAAAATT
TTTTTTAANTTCNAAAGACCTGGGGCTTTTTTTTTTTTTTTTTTCCCGGGNGNNCCCCNTAAAATTTCCCCT
TTTTTCCCANGGGATTTTGNAAAGNAAAAG

SK21

CCNTTTTNTGAGGAGGTTGACGGCTCTGCTGGGGCTTTTTTGAGGTTGCTGAGAACATTGACAACGCGGAAG
TTGCCTCTTTCGCTGAGGAGATCCCCGCTGACGCCGTCAAGGTCGTTGACATCGCTGCCGAAGACCAGGCTG
TCGCTGAGGAAGCTTCCGCTTTTGCTGCAAACTCGGATCCCAGNGCACTTCGTTGAAGGGGTGAGACATCNA
CGTCAAAAACCCGCCGCCNCCTGAAAAATCGCCGTTGCGCCGTCAAGGNCCCCACTTCCTTGAAACCGTGAA
ATTTGTTCCCGTGCCAACCCCTTCNCCNATTCCGTCCGNGAGGAAAACCTTCCCTTAACTCCCCCGNAAAAT
GGATGAACACCCCNTGCTNCCTTAAAAACCAAAATTTTTCCAAAAAAAAAATAAAAAAAAAATTTAACTTTTT
ATTTTCAAGNACAATAATGACTGNGNTTAAAGCCATATTTGGCCCATNGTNTTTATNACCAATATTGTTCCA
ANCCTTNAAACTNTTCCACCCGGCCCN

SK29

CAGCGCTGTAGCGCCATAAAAATCAGTACCTATATATGGCAATTGAAGGGTAAGTTACTACGATTCTAACGA
TACCTCTAGCAACAAAATCTTACATATACATGCACGAATTGAGGAAATTCCAAATAACCAAATCTATAATAG
CCGTTAAGTTCAAACATCGGCAATGATCGGCCCACATCGGATATCTTCGGGCCCTAACTATACCATTTGTGT
TGTAAAGCGATCCTTTATGTTAGCTTTTATGTTTAGTTAATTTGTTTATAGGGATAACGTTACACCAGGGGG
TCATGCGCTGTTCGATAGCCGCCGTAACCGGCCCGGATTAGGCCTAATTCTGTTAATGATAATTAGCATCGG
AGTTCAGAAATTGCAGGTGGCAATGGCGGCGGCGAGATGAAAAACAATTATAGATTTGTTGTTNGTTTCTCC
AAGTTTGGTCTGGTGTTATAATTGAATTTGGAATTTGAATATGTTGTTGGTCTGTGCCCAAAAGCGTTGTTT

AGACCTTTCGGTTTGCTCAGCCTGATGAGATTGATCGGTTAATGGTTTCGAAATGTTGGGGTCTCCGAAATT
GGAAATGCTNAACTGGGTTACCTAANTTTAGCAAATCAATCCGCAACCGTTTTGAAGTNTTTATTNTAACCC
TNNCTCAAACGNCTTANAGGAAACNTTNTNTGGGCAAAGATGAAATAAATTAGCTTNTTTAAAAANTTGTGG
TTGGGGAACGTTATNCCCATTNTACCAN

SK30

ATGAAACTGGTTCTGTTCGCTTCCCTGATCGTCGTGGCCGTCGCCGCGCCCCGCAATGGCTTGGTGGTGCCT
GTAGCCGCCGGACCCGCTCCCGCTCCCGCATTCGATGGACCAATCATGGCTCCCGTATTTGAAGCACCAATC
AAACCCGTCCCTACGCCCGCTTTTAACCCGATACTCTGCCCACCTTCTGGTATGCTCCGCAAGGTTGACGTC
AGCTCTGCCTGCGCTGGTGTCATTGCTCCCCAACCCGTCATTGCCGCCCCTGCCGCCCCCTCCCCCAGCAAC
CCATCTCCTCTGGTCCAGATCGTTCTGAACATCAACCAGGAAGCTTCTGCTGCTCCCGTCGTCGCCCCCGAG
CCCGTCGTCGCCCCCGAGCCCGTGCAGGTTGTTGAGACAGCTCCCATCCCCGTGGAGCCCGTCCAGGTTGTG
GAGATCTCTCCTGAGCCCGTGCAGGTTGAGGAAGCTGCCCCCGTGCCCGCTGAGCCCGTCATCATTGGAGAG
CCTGTGCTCCCCTCCCCTGCCATCACCCTCCCTGAGGAACTCAACTAAAGATACGATGTATAGACACATTTA
TTCCAAATGAGACCTGATCAATAAACGTTCAATAATTATTAAAAAAAAAAAAAAAAAACTNGAAGTCTTTTA
AAGNGGCGGGGGCCCTTGNTTTTCNCCCGGGGGGGGNCNGGGAGGGGNCCCAANCCCCTTTNNGG

SK35

AATAAATAGTTACTAACTTGACAATGATTCATCATTAGAAGATGGAAAGGTTAGTCACACGTATTTATCGGG
ATTGTTCGACACATTACGACCCCAGCAGGTACGATTCGCGACCCCGTCGCGGCAGGCGTCAATTCTAATAAA
AACGCGGGAGTACATCATTTATTAAAAAAATCTCTTAAGTTAGTAATCCTTGCAAAATGTATCTCAATTCAC
TTAGTAATACCGACAAATAATAAGGTTATACGCGTCTTTGTGGTTAAAAGATACCATACAAATTCTTACAT
GAATTGCAAATTTTCTTTCGATAGTTTTCGTGATAACTCCAGTGTTCTGTGTTCGCTTATTGCGCTATTTAC
ATATATACCACCATATACTTACTACATATAACACTGGTCCTAAGACTTATTATTGCGCCATTGAAAN

SK39

CAGACATCATNTTGNGGTTAAACCAAAAATANCAAAAAAATANCAAAATGAAAATCGCCGTGTTCTTCGCAT
TTNCACTCGCTTTCNCATCTGTTCAATCCTCTCCCCTGTNGGGNGGTTNGATAAGCGGTATATCGAANGGAG
TTAGNGGTGTNTTAAATAATGCCGNTGATGCAGTTGGCNCTGTTGGTAACNCTGTAGGAGGTGCTGTTGATA
ATGTAGGCTCCGCTGTAGGAGGCGCTCTCGGNAATGTTGGCAGCGCTGTAGGAGGCGCTCTCGGTAATGTTG
GCAGCGCTGTAGGAGGCGTTCTTGGTAATGTNGGCAACGCTGTAGGAGGCGCTGTTGATAATGTTGGCAACG
CTGTAAACAATGCTGTTGATAATGTTGGCAACACTGTAGGAGATGCTGNTGATAAT

SK45

GCGCGCCTTTTGGNCGACACTAGGGGATCCAAAGAATTCGGCACGAGGTTTNATATTTAGTTNGGTNTTTTT
GGCTTTTAATTTTAAGNGCAAAGGGGNTAAGGTTCTTAAATCTGTGAGGTATTCATTGGCGGCTGTGAATGG
AGCATTTTTGCTAACAGGACTTCTATTATTCTTCGTCGGCATCGCCGNGCTGGGGGAGTACAAGAACTGGGA
GGGGCTGATCACCGGGCGGNTCTTCCTCTTGCTAAAATTCGNAGGGGGANCCGAGCTATCAAAATGGGTGGG
TNCCGGCCCCGGGTTTACCCAACTTATACAAACNCTTNTAATTTGGTGGCNGGGAAAGGGGGCCGNTTTTGG
GGAACNTGGGCTTTNAAAAAACNGAAAAGCCAAACCCGCCTTTNGCTGGACCACCANTCNTNGNAAGGAAAA
CAGGCCATCGATGGGGGCCAGCCCAAACTGGGCCAACANANGGTCCAAATTACCNAAACCGGGGGACCAAAT
GCAAAANGGANACCNAAAGCCGGNGGGGGGGGGGGCCCCCCCGAACGGGGCATGGGAAAACCANCCNAGGGG
CTGGTGGCCCAAGGGCCACCGGGAATTTATNCCCCN

SK60

ATGAAACTGGTACTTTTCGCTTCCCTGATCGCCGTGGCCGTCGCCGCGCCTCGCGCTCCCCTGTTTGATGTA
CCCATCAAACCCGTCCCCATGCCCGCTTTTGACCCAATACTCTGTGGACCGGCCGGTTTGCTCCGCAAGGTT
GACGTCAGCTCTGCCTGCGCTGGTGCTATTGCTCCCCAACCCGTCATTGCCGCCCCTGCTGCCCCCTCCCCC
AGCAACCCATCTCCTCTGGTCCAGATCGTTCTGAACATCAACCAGGAAGCTTCTGCTGCTCCCGTCGTCGCC
CCTGAGCCCGTCGTCGCCCCCGAGCCCGTGCAGGTCGTCGAGACTGCTCCCATCCCCGTGGAGCCCGTCCAG
GTTGTGGAGATCGCCCCTGAACCCGTCCAGGTTGTGGAGGTCGCCCCCGCACCAGCTGAGCCCGTCATCATC
GGAGTGCCCGTCATCCCTTCCCCCGCCATCACCCTCCCCGAGGAACTCAACTAAGCGATAGACAAAACTCGA
TTCTTCATAAATAATTAAAAATTCAAAAATCTNTATCTACAACTCTGTTATTTAAGATGAAAAANAAGGGCT
GNACTGGCTTTTCCGGCAACAACCTTCTTGNGGAACTACCTAAAGACATCGAATCATGNGACCTAATAAANA

ANGTTCTTATTTTTAAAAAAAAAAAAAAACTTNGAGGTNTTTTAAGGGGGGGGGGCCCCTNNTTTTTNCCCN
GGGGGGGGGGAGAGGGGCCCAATTCCCTTNNNNN

SK61

AGAACACCATGTATGTTGTTCTAGAAGAGTATTTGACTTCTTCACATGTTAAAATTGTTCGAACAAAATGGC
GGTTATACATAACCTATTACATGGAGTTATGTTTTTTACAAGCTTTAATAACAAGGTTTTTCGGAAGTTTTA
CAGCTTAAGTGACTTAACAGTAATCTCGCTATCAGTTATTGTTTAAAAACAATGTTCCTTTTTGTNCTTATT
AGATTTATGTAACGCGTATAAATTATTATCTNGNGATTTTATATGNGAGGCAAAATTTTNNTNTNACNTANN
TNNGTTANGTNNNTTTACCTACCAATAATTAANTNGTTTGGGNCTTTANTNCGTTTNNTTTNGNTGNNNTTA
AGNAAAANACATCCCNNTTGGCCATGGTTGGTTTTTTTANTGANGAAGCTTCCCTTGNCCGTGGGGGGGGTT
TTACCCGGACACTNANTTTTTNTATNTTAAANCCTNAAATTNGGAGGGCCNGGCCTATAAATTGGGAGGGCC
AANGNGCCTTCCACGGTTAAANTCCTGTCCCNCCNAACCGNGTTNACCAGATTTAAAGAAAAANAATCATCT
TTGGGAAGGTCTTACACGGGGCNAGGGNTGGCTTTTTTTTTTTTTTAAAANAATTNAAAAGGCCGGGGTTTT

SK73

CNCCATTTNNCNNCACTNGGGGNGCCAAAGAATTCGGCNCGAGTCTCTAACNNTNGNNCATTTTNNTTTAAA
TTTCGTCGACTCTTTTATGGGGGNANNTTTTAAATTTNTNNGGGTTGNAATATANTTATAAATCAGGTAATT
TTAGGNCGATTTTAATTCCTTATCATTTTTATATCAACGNTTCACATTNCACCNCAATCATGAGTTCTGGTG
GCNCTCCCGTGNATGNCGGCGGATTAGTANAAGGCCTCAANAAGGACCACCCCCAAGAGAATTCCATNAATA
CGGGAAACTAAACTCCTTTGGGGGGCACTCAACCCCTCGGGGNTTGCATNCATACAAATNGAAAACTTGNCG
GAAGCTTNGGATGCTNTCCCCCATGAACGGGATCCNATGCTAGGCCCCCCCTCCNCNANAAAAATTTAGCCA
CGAGATCGGCCCCCCNCGGGNGGCTGNTTTCCTGGANGACCCCGCGGCGNNACTTNAANGGNCGNGGATTCG
GGGCCCCCCCCCGGGGGGGGAAAAAGAATCCTTCTCCCCTCCGGGGGGGGCCGGCCTACAATATTAACCACCC
AGGGGGGGGGAGGACCNGGTN

SK82

GGAATGCTTGAGCCCTCAGCTCGCTGGCTTATGACCAGCACAGAACAAATATTTATTTTACGTTGGTATTTG
TTCTTATCGTTCGTGCAATAAGATTTGTGTCTTACAACCGCACCAATTTCTAACCACAAGATATCTGAAAAA
TACGCATGCTTCTGGAATTAAATTCGAATAATGCGTCATGCCCATAGCGACTCCAGTTGATTACTACTCTGT
GCCCGCGTAATATAATTGTGTGAGCGTGCGGTCTGCGGCGCTCGCTCTAGTGTTACATGTTGACTTGCCATG
TTTTGAAATATTTTTGTAATGTAAGAGATGGTACATTAAAGTATAATATTTTTTATGTCAGATTTATTTTAT
TCATAATTTTTAAGTAAAATGAACTTAATTGTTATGCATTGTTGACATAATGAAAGATAATCGTAACGTGCC
TCATATGAACATTGATATTTTATTTTATTTTATGCCAATATTNGNAAAAGATAGATTTAAGTAATTACGTAA
TGATTGGTAATAATAATAATTTATCGCGATGTTCATTTATTTAAATATAGTCAATATCTAAAAGTTGCAATA
ATCCTTTGTCNGGGNGACTACGAGAATAAATATTGNATTATATGAGAACATGATAGTTTGGCGAGAGAATAA
TCGGTTGGNTGNCCCTAATNANANACCTAATNTNTAANGAANACCGGGNGNNGGGGGTAACCCNAAAACTNG
AATTGTGCCTCCCNCNNCCTTGTTTTTTTTTGTN

Fig. 1.7. SK nucleotide sequences and their BLAST search results showing access numbers of the top matching sequences, putative function, organism, score and E-value for each sequence, the nucleotide sequence alignment (obtained from BLASTN) or amino acid alignment (obtained from BLASTX), and the frame used in deducing the amino acid sequence. Each SK is shown in separate page.

SK01

```
CACGGAACCAGTGCAATCCTACCGCTATGAGAAGTTCTTCCACGACCAGATCAGTGCTAAGAAGAGGGACTA
CTCCTACCGCGTCTTCCGGAAGGTATCGCGGTTGGCAGCGGAGGGCGTGTACCCGCAGGCGCTGGAGGGACC
CGACAACCACCGCGTCACCGTCTGGTGCGCTAACGACTACCTCGGGGCCTCGCGCCACCCTGTTGTCCAAGA
TGCTGCAGTCAATGCTATCAAGTCATATGGGTACTGGAGCAGGAAGGTACCAGGGAACATTGCAGGAAACTC
ACAGATGACTGAAAAATTGGAGGCTGAAATAGCAAATCTGCACAAGAAGCCTGCTGCTTTAATATTTAGTTC
ATGTTTTGTTGCAAACGATGCAACACTGTCTACTTTAGCAAAACTTTTGNCGGGTTGGTTAATTTATTCTGA
TGCTGGAAACCATGCCTCGATGATACAAGGGATTCGAAACAGTCGAGCCCCAAAGCATATTTTCAGGCNCAA
TGACCCCAAATCATTTTAAGAGAATTGTTGGGTTCAATCGNCAACTGGGGGTACCCCANCTTAGTTCGGGGT
TTGAAAACTTGTNCCCCTNTNTTGAGNGGGNGCGGATTTTGTNCCTTTTAAANGNAAAAGGGGGGATTTTAG
CNCCATGGAGTTTTGGGGGCTCTTTACATTTTGTTGGATGAAGGGCCCATTGCTNG
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Sequences producing significant alignments:          Score (bits) E-Value
gi|31207831|ref|XP_312882.1| ENSANGP00000014729 [Anopheles ... 197 2e-49
gi|19527643|gb|AAL89936.1| SD01515p [Drosophila melanogaster]  191 1e-47
gi|17137420|ref|NP_477281.1| CG3017-PA [Drosophila melanoga... 191 1e-47
gi|4433540|gb|AAD20805.1| 5-aminolevulinate synthase [Limul….. 189 6e-47
gi|51704017|gb|AAH80965.1| Unknown (protein for MGC:79710) ... 176 3e-43
gi|4433546|gb|AAD20808.1| 5-aminolevulinate synthase [Sepia... 176 3e-43
gi|49522083|gb|AAH74129.1| MGC81838 protein [Xenopus laevis]   176 6e-43
gi|37747732|gb|AAH59988.1| MGC68700 protein [Xenopus laevis]   175 9e-43
gi|763098|emb|CAA27223.1| ALA-synthase [Gallus gallus] >gi|..... 174 1e-42
gi|63608|emb|CAA26595.1| precursor (aa 1-635) [Gallus gallus]  174 1e-42
gi|202860|gb|AAA40724.1| delta-aminolevulinate synthase pre .. 172 8e-42

Frame = +2
Query: 14  QSYRYEKFFHDQISAKKRDYSYRVFRKVSRLAAEGVYPQALEGPDNHR-VTVWCANDYLG
           ++++YE FFH+QI  KK+D+SYRVF+KV+RLAA+G +P+ALE     R +TVWC+NDYLG
Sbjct: 159 RTFQYEDFFHEQILRKKQDHSYRVFKKVNRLAADGQFPRALEYSWGERPITVWCSNDYLG

Query: 191 ASRHPVVQDAAVNAIKSYGYWSRKVPGNIAGNSQMTEKLEAEIANLHKKPAALIFSSCFV
            S HP V+ A  +A+++YG  +     NI+GNS   E LE  +A LH+K +AL+F+SCFV
Sbjct: 219 MSCHPEVKRAVADALETYGTGAGGTR-NISGNSMNHENLERRLAELHQKESALLFTSCFV

Query: 371 ANDATLSTLAKLLXGWLIYSDAGNHASMIQGIRNSRAPKHIFRXNDP511
           AND+TL TLAK L G  I+SDAGNHASMIQGIRNS  PKHIFR NDP
Sbjct: 278 ANDSTLFTLAKALPGCHIFSDAGNHASMIQGIRNSGVPKHIFRHNDP324
```

SK11

GCGAATGGTATGCAGGGGTCGGGAGTCCGCGCCCAAGGCGCTTATGATGGCGACGGAGCCGTCGAGGTCCCC
CGCCCAATTGGGACGAGGGGCAACATAATATGGCTCCGCCGCCACTGCGATATCAATGCCCCACTGCGCCAG
CGACTGAAGGAAAAGATCCTGTGCGCCGGCGCAGTGGTTCAGGTTCCCCTGGAGGAGGAAGTGTGGACGGAC
GTTATCCATTAACTGGGTCGTCCATCCGTTCCTCCTCACAGGGTTGCGCCCGTGATGNANGTGNCCGCATTG
GGCACTTTCCCCAATGCGGCCATTTGCTTCTTGATAAGGGCCGGGGGAGCACATCCCCGGCTGCCCATTTGG
TGGGCGGCACCGCGACCGAGCTCGGCGCATATCGCGCACCTGGCCGGCTCTTTGCACACCGCTGCCTTGTGC
CCGGGCATCGCGCAGCGGTAACATAAACCGCTGCGGTCCGCGGGGCACATGCACACCGGNCTTGTGTGGGCG
AGTGCTAGGCACGGTAGCACTTNANTGGTCGTGCTTCAACTTGACCACCGTGGGCCAACTTCATTCCANCAG
GAACTTNTGGTCCTGGNGGGAACAGCCTTGGNCCCCCTTCAGNGGNANTTTAGACCCCCTTTNTTTTNGCCC
CNTTTTGGCCCNTTTTACCCGGTTTGGGGCGGGGCCCCTTTAACCTGGTTTTGGGGGTTGGACNAGGCCNNN
CTTTCCTTCGGGCTTCCTNNCCCTAAGNGNG

Sequences producing significant alignments: Score (bits) E-Value

gi|22004007|dbj|BAC06454.1| reverse transcriptase [Papilio ..	73	7e-12
gi|22004001|dbj|BAC06450.1| reverse transcrpitase [Papilio ..	73	7e-12
gi|28569894|dbj|BAC57926.1| reverse transcriptase [Bombyx m..	72	1e-11
gi|22004010|dbj|BAC06456.1| reverse transcriptase [Papilio ..	72	1e-11
gi|22004004|dbj|BAC06452.1| reverse transcrpitase [Papilio...	72	1e-11
gi|2662336|dbj|BAA23655.1| ORF2 [Bombyx mori]	71	2e-11
gi|22004023|dbj|BAC06464.1| reverse transcriptase [Papilio ...	71	2e-11
gi|7511775|pir||T18195 gag protein - silkworm >gi|2055275|d..	70	6e-11
gi|22004020|dbj|BAC06462.1| reverse transcriptase [Papilio ..	69	1e-10
gi|7511782|pir||T18196 pol protein - silkworm >gi|2055276|d.	69	1e-10
gi|22004017|dbj|BAC06460.1| reverse transcriptase [Papilio ..	69	1e-10

Frame = -2
Query: 201 LLQGNLNHCAGAQDLFLQSLAQWGIDIAVAAEPYYVAPRPNWAGDLDGSVAIISALGADS
LLQ N+NHCA AQDL +QS+A+W IAV +EPYYV R +W GD D VA+I A S
Sbjct: 5 LLQANVNHCARAQDLLIQSMAEWSTQIAVVSEPYYVPNRDDWVGDEDSLVALIVPRSARS

Frame = -3
Query: 515 CLALAHTXPVCMCPADRSGLCYRCAMPGHKAAVCKEPARCAICAELGRGAAHQMGSRGCA
C AL H C DRSG CYRC GHK+A C C ICA GR AAH G + CA
CHALGHVSARCPSSVDRSGECYRCGQTGHKSAGCALTPHCTICAGAGRPAAHVSGGKACA

SK16

GGGGCGTTGGCTCCCACTAATTCTCATCCTTATTTCGCTGGTCTGCTGATCTCTCTCCTTGGTGTCACCGGT
AACTCAGTATGCGGCTCTACCCTGCTGTCTCAAAACCGACTGGTGACCGCAGCTCACTGCTGGACCGACGGC
CGCAACCAGGCCACACAGTTCCTCGTCATCCTCGGCTCCAAGCTCCTNTTCTTCGGGGGAACCCGCATCCCA
ACCTNAAACGTNATTATGCACCCCCAGTATTTNCCCGCCAACCTCAANAANAACATNGCTATGATATACCTG
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TTTGTCGGCAACTGGGCTNTAACTNCCGGTTTTGGCAGGACTAGCGACGCTNAAGCCGGNGCTTNAACGGAA
GNGAGCCACGTGACCCTGCAGGNGATCANCGTGGCTNAATGCCANGCCGGGTTCGGCAACAACTTCGGGGTC
CANANCACCATNTGCACCAACGGCGCCNGNGGNGTCGNATTTGGGGNGANATTCNGNGGACCTTTTTACTTA
ACCGGGGGGGGACGCACTGTTNTTNTTGGGGNANATTCTTTGGGGCCGGGNGGCTGCAAGTTGGTTTCCTTCG
CTTTGNTGGGAACAGGTTAAAANTTTTTCCCCGGCATGTAACTTTTCNGGCCCATTCCTTTTNTTTTGGNAA
AATTGGGGCCNGACNTTAAAAGAAAAN

Sequences producing significant alignments: Score (bits) E-Value

gi|2463064|emb|CAA72952.1|	chymotrypsin-like protease [Heli.	142	8e-33
gi|29501764|gb|AAO75039.1|	chymotrypsin precursor [Spodopte...	142	1e-32
gi|2463076|emb|CAA72958.1|	chymotrypsin-like protease [Heli.	139	6e-32
gi|2463080|emb|CAA72960.1|	chymotrypsin-like protease [Heli.	136	4e-31
gi|2463092|emb|CAA72966.1|	chymotrypsin-like protease [Heli.	136	5e-31
gi|7248890|gb|AAF43709.1|	chymotrypsin-like protein precurs..	136	5e-31
gi|2463078|emb|CAA72959.1|	chymotrypsin-like protease [Heli.	135	9e-31
gi|8037824|gb|AAF71519.1|	HzC20 chymotrypsinogen [Helicover..	134	2e-30
gi|8050592|gb|AAF71716.1|	HzC4 chymotrypsinogen [Helicoverp..	134	3e-30
gi|2116576|dbj|BAA20136.1|	serine protease precursor [Bomby..	133	4e-30

Frame = +1

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Query: 1   GALAPTNSHPYFAGLLISLLGVTGNSVCGSTLLSQNRLVTAAHCWTDGRNQATQFLVILG
           G++    + PY AGL+I++      SVCG++L+S NRLVTAAHC +DG   A  F V+LG
Sbjct: 61  GSITNIANVPYQAGLVITIF--IFQSVCGASLISHNRLVTAAHCKSDGVLTANSFTVVLG

Query: 181 SKLLFFGGTRIPTXNVIMHPQYXPANLXXNXAMIYLPYNIFXXGSIQPISLPNSSELXDS
           S  LFFGGTRI T +V+MHP + P N   N   +    ++     IQPI+LP+  EL +
Sbjct: 119 SNTLFFGGTRINTNDVVMHPNWNP-NTAANDIAVLRISSVSFSNVIQPIALPSGDELNNL

Query: 361 FVGNWAXTXGFGRTSDAXA-GAXTEXSHVTLQXIXVAXCXAGFGNNFGVXXTXCTNGAXG
           FVG  A   GFGRTSD+ + G   + S VT+  I  A C A +G+ F      CT+GA G
Sbjct: 178 FVGANALASGFGRTSDSGSIGTNQQLSSVTIPVITNAQCAAVYGSGFVHASNICTSGAGG

Query: 538 V-XFGXXFXGPFYLTGGDALXXLGXILWGRXA--ASWFPS 648
                 GP  +   +    +G   +G  A  A+ FP+
Sbjct: 238 KGTCNGDSGGPLAVDSNNRKILIGVTSYGAQAGCAAGFPA 277
```

SK17

GGCNCGAGCAAAAACCGATATTGTGTTGTAATTTGCTGGTTTAATTACTTTTTTTCCCTTCCAACAAGGGCA
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GGCGGAGCAGGCTGAACGATATGACGACATGGCGGCCGCGATGAAAGAAGTCACGGAAACCGGCGTCNAGCT
GAGCAACGAGGAAAGGAACCTTCTTTCCGTTGCTTACAAAAACGTGGTGGGCGCTCGGCGGTCGTNATGGCG
CGTCATCTCCTCCATTGAACAGAAAACCGAAGGATCGGGAAGGAAAACAACAGATGGCAAAAGAATATAGGG
TTAAAGTAGAAAAGGAGCTGAGAGAAATCTGCTACGATGTCTTGGGTTTACTTGACAAGCAC

Sequences producing significant alignments: Score (bits) E-Value

gi|48097086|ref|XP_391841.1|	similar to ENSANGP00000009311	139	2e-32
gi|33415278|gb|AAQ18147.1|	14-3-3 protein [Branchiostoma be...	119	9e-36
gi|10719663|gb|AAG22081.1|	14-3-3.a protein [Fundulus heter...	115	3e-33
gi|47938859|gb|AAH71323.1|	Zgc:55807 protein [Danio rerio]	117	7e-33
gi|47086531|ref|NP_997922.1|	Unknown (protein for MGC:55807	117	2e-32
gi|34452075|gb|AAQ72494.1|	14-3-3G2 protein [Oncorhynchus m.	119	1e-31
gi|34452073|gb|AAQ72493.1|	14-3-3G1 protein [Oncorhynchus m.	119	1e-31
gi|17530147|gb|AAL40719.1|	14-3-3 product [Meloidogyne inco	119	1e-31
gi|49227280|ref|NP_998187.1|	3-monooxygenase/tryptophan 5-m.	119	2e-31
gi|47227811|emb|CAG08974.1|	unnamed protein product [Tetrao	119	2e-31

Frame = +2

```
Query: 104 MSVDKEELVQRAKLAEQAERYDDMAAAMKEVTETGVXLSNEERNLLSVAYKNVVGARRSX
           MSVDKEELVQRAKLAEQAERYDDMAAAMK VTETGV LSNEERNLLSVAYKNVVGARRS
Sbjct: 1   MSVDKEELVQRAKLAEQAERYDDMAAAMKAVTETGVELSNEERNLLSVAYKNVVGARRSS

Query: 284 WRVISSIEQKTEGSGRK 334
           WRVISSIEQKTEGS RK
Sbjct: 61  WRVISSIEQKTEGSERK 77
```

Frame = +3

```
Query: 303 LNRKPKDREGKQQMAKEYRVKVEKELREICYDVLGLLDKH 422
           + +K +  E KQQMAKEYR KVEKELREICYDVLGLLDK+
Sbjct: 67  IEQKTEGSERKQQMAKEYREKVEKELREICYDVLGLLDKY 106
```

SK19

ATAGAATTGAAGTCTACACAAGAAGCAGCAAGATTGACTCACAAGGTTACAAATGGACATCAGACGGCTCTG
GCATGTATGAAATACAAGAAAGCCCGATGGGAGTGCCAATTGGGTACAAAAATAATAGTNCACTTAAAAACT
GNCTGGCCGAGAATTTGCTGATGATGACNCAGTGCAAAACATAATAAAGAAATNCNGCNACTTTGGGAGCAG
TCCTATNTTTTTAAATGAAACACAAGTTAACTNTNTTAAGCCTNTTTGGNTGNTGGAACCCAAAGAAGGTAC
CATGGGANCACCNCATAGAGTTCTNTAAATATATCAAGCCANGGCTTATGACAAACCCCAGATTTACCCTTC
ACTACCAAACTGATGCTCCCCTCAGCATCAGATNCATTTTATATGTGCCGGAAGGCAAACCTGGTCTCTTTT
GAACTATCGCGAGATACCGACGTTGGAGTTGCCCTGTNCTCAAGAAAGATATTGNTCAAGAGCAAGGNTTGA
AAACATTTTGNCTAAANGGGTGGAGAATTTGTNAANGGGGTGGGGGTTNCCNAAAAATTTCCNTGGAATNTT
TATNGGGGGGCTTTTGGGNNAACAAGGGCCCCTTNNTTTTGGAAACTGGNAAAACTGGGTCNTTNCNAAAAC
CCGGTTTCCTTTAAANTTTTNGGANNGGGATNNGGCCNAAANAAAAAAAAACTNGGGGG

Sequences producing significant alignments:	Score (bits)	E-Value
gi|31204913|ref|XP_311405.1| ENSANGP00000016646 [Anopheles ...	89	9e-21
gi|13385998|ref|NP_080784.1| TNF receptor-associated protei...	84	9e-17
gi|13879408|gb|AAH06685.1| Trap1 protein [Mus musculus]	84	9e-17
gi|17511976|gb|AAH18950.1| Tumor necrosis factor type 1 rec...	83	2e-16
gi|7706485|ref|NP_057376.1| tumor necrosis factor type 1 re...	83	2e-16
gi|37589015|gb|AAH01455.2| TRAP1 protein [Homo sapiens]	83	2e-16
gi|1082886|pir||A55877 tumor necrosis factor type 1 recepto...	83	2e-16
gi|2865466|gb|AAC02679.1| heat shock protein 75 [Homo sapiens]	83	2e-16
gi|3273383|gb|AAC24722.1| TRAP1 [Homo sapiens]	83	2e-16
gi|21752190|dbj|BAC04139.1| unnamed protein product [Homo s...	83	2e-16

```
Frame = +3
Query: 3   RIEVYTRSSKIDSQGYKWTSDGSGMYEIQESPMGVPIGYKNNSXLKNXLAENLLMMTQCK
            R++VYTRSS+  + G KW+SDGSG +EIQE+   V IG K    LK    E      + +
Sbjct: 233 RVDVYTRSSRAGAPGLKWSSDGSGTFEIQEAE-NVAIGTKIVIHLKADCRE-FADEDRIR

Query: 183 T**RNXATLGAVLXF*MKHKLTXLSLFGXWNPKKVPWXHXIEFXKYIKPXLMTNPRFTLH
               R  +       F    +   +       PK+V      EF +++     T PRFTLH
Sbjct: 291 EVIRRYSNFVGSPIFLNGKQANQIQPIWLMEPKQVTPEQHNEFYRFVGNTFDT-PRFTLH

Query: 363 YQTDAPLSIRXILYVPEGKPGLF 431
            Y+TD PLSIR +LY PEGKPGLF
Sbjct: 350 YKTDVPLSIRALLYFPEGKPGLF 372
```

SK20

AGGACACCGACAGTGAGGAGGAGATCCGCGAGGCGTTCCGCGTGTTCGACAAGGATGGCAACGGTTTCATAT
CGGCCGCCGAGCTGCGCCACGTCATGACCAACCTCGGCGAGAAGCTCACCGACGAGGAGGTCGACGAGATGA
TCCGTGAAGCCGATATCGACGGCGACGGACAAGTCAATTACGAGGAATTCGTCACCATGATGACGTCGAAGT
GAGCCGCCGGCTAGTGTGTGTGTAAAAAGCAGAGAATTTAAATATACATTTTGTTTCATGACACACAATATT
ACCATCGTATTGGACCTGCATTTCTCTAGTGTTTGCGATAAATTAAATATTGTCATCGTTTCAGTTTAATGA
TGTACTACTTGTAACGTACGCGCGCCGCCTCCGCCTTCTCTTTTTATTTAGA

Sequences producing significant alignments: Score (bits) E-Value

gi|31201603|ref|XP_309749.1| ENSANGP00000012700 [Anopheles ... 144 8e-34
gi|115522|sp|P11121|CALM_PYUSP Calmodulin (CaM) >gi|71670|p... 144 8e-34
gi|27065792|pdb|1MXE|B Chain B, Structure Of The Complex Of... 144 8e-34
gi|2464957|emb|CAA05092.1| calmodulin [Branchiostoma lanceo... 144 8e-34
gi|51557667|gb|AAU06473.1| calmodulin [Culicoides sonorensi... 144 8e-34
gi|49035528|sp|Q8STF0|CALM_STRIE Calmodulin (CaM) >gi|20152... 144 8e-34
gi|115526|sp|P21251|CALM_STIJA Calmodulin (CaM) >gi|71669|p... 142 2e-33
gi|17564542|ref|NP_503386.1| calmodulin (16.8 kD) (cmd-1) [... 142 2e-33
gi|115518|sp|P02595|CALM_PATSP Calmodulin (CaM) >gi|71671|p... 142 3e-33
gi|33243604|gb|AAQ01510.1| calmodulin [Branchiostoma belche... 141 4e-33

Frame = +3

```
Query: 3   DTDSEEEIREAFRVFDKDGNGFISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVN
           DTDSEEEIREAFRVFDKDGNGFISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVN
Sbjct: 106 DTDSEEEIREAFRVFDKDGNGFISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVN

Query: 183 YEEFVTMMTSK 215
           YEEFVTMMTSK
Sbjct: 166 YEEFVTMMTSK 176
```

SK22

AGAGGTGGTTGTGTGAATCAAAATGAAGTCCTTAGTGCTAGCTGTTGTCAGTTTACTGGCAATTTCATCAAT
AAATTGTGAAGTGTTCTTTGAGGAGAAATTCCCTGATGACTCATGGGAATCCAACTGGGTGTACAGTGAGCA
TCCCGGAAAAGAGTTCGGCAAGTTCAAGCTGACTGCTGGAAAGTTCTACAATGACCCGGAGGCAGATAAAGG
TCTTGCAGACGTTCCGAAAAACCCNNCGGTTCTACGCGTTGTCCCCCAAGGTCAAACCCTTTAANAACCGAG
GGCAAGCCCCTGGTGGTGCAGTTCTCCGTGANGCACGAGCAGGACATCGACTGCGGCGGCGGCTACCTGAAA
GTGTTCGACTGCAAGTTGGAGCANAAGGACATGCATGGCGAGACCCCCTACNAGATTATGTTCGGNCCTGAC
ATNTGCGGTCCTGGTACCAAGAANGTGCACGTGATCTTCAGTTNCAAGGGGCAAGAACCACCTNATNAAGAA
GGACATCCGCTTGCAAGGGTGATGTCTACCCCTCACTTTGTACACTTTTGATCGGGGAAAGNCCCGNCAACC
ACCCTATTNAGGGGGCTTATTTGGGCCAATTAAAAAAGGTTTAATTTCCCGAAAAAGCTTNGAAGGCCCCAC
CNGGGGAACTTTCCTTTTCCCCCCCAAANAAAAAATTNAANGGGNCCCCCCNAAAGCCCCAAAAAAANTNCT
TN

Sequences producing significant alignments: Score (bits) E-Value

gi|31559109|gb|AAP50845.1| calreticulin [Bombyx mori] 134 5e-60
gi|28804517|dbj|BAC57964.1| calreticulin [Bombyx mori] 134 5e-60
gi|17826933|dbj|BAB79277.1| calreticulin [Galleria mellonella] 125 2e-58
gi|25527292|gb|AAN73309.1| calreticulin [Cotesia rubecula] 119 7e-48
gi|48101589|ref|XP_392689.1| similar to calreticulin [Apis 117 3e-47
gi|18389889|gb|AAL68781.1| calreticulin [Anopheles gambiae] 117 6e-45
gi|31208299|ref|XP_313116.1| ENSANGP00000012895 [Anopheles ... 117 6e-45
gi|18568312|gb|AAL76026.1| putative calreticulin [Aedes aeg... 116 1e-43
gi|24645441|ref|NP_524293.2| CG9429-PA [Drosophila melanoga... 118 3e-43
gi|6063416|dbj|BAA85379.1|calreticulin [Drosophila melanog. .. 118 3e-43

Frame = +1
Query: 274 PLXTEGKPLVVQFSVXHEQDIDCGGGYLKVFDCKLEXKDMHGETPYXIMFGPDXCGPGTK
P EGKPLVVQF+V HEQDIDCGGGYLKVFDCKLE KDMHGETPY IMFGPD CGPGTK
Sbjct: 83 PFSNEGKPLVVQFTVKHEQDIDCGGGYLKVFDCKLEQKDMHGETPYEIMFGPDICGPGTK

Query: 454 XVHVIFSXKGQ 486
VHVIFS KG+
Sbjct: 143 KVHVIFSYKGK 153

SK28

GTTCAACATGAAGGGTTTTGCATTTGTTTTGTTGTGCGCGCTTGTCGCCGTCCAGGGCCGCAGCACCGCGGT
TCAATCCGCAAGCCGCGAGCTCGCCACCCTCGGTGAGAACCCCTACGTGGTCCACCTCCGCCTGGCGGTCAG
CACCAGCGGTCTCCTGAAGACCTGCGCCGCGTCCGTCATCTCCCCATCATGGGTCCTCACTGCTGCCAGCTG
CCTTGAANACAACCGTTACGTCTGGATCCGTTTCGGAGCCGTGAACGTCGCCAACCCTACCCTGGTGTTCGA
GGCTGGTAACGCCGGCGTCAGAGTGCTCAACAACCTTGGTCTTTACACCGCCAACAACAGAGCCGTCGATTT
CTCACAGGCAGCTACCATCGCTCCCGTCGCCCTTTCCGAAGCTGGTGTCTCAGACTCCGGTAAACTCTGCGT
TTTCGGAGCCAACAGCGAAGGCGGCCCCGGTGAGCTCCTGTCTTGCGCCACCGTTGGTCTCGAAGCCCAAGA
NGATGGTTCCGTCATCATCAGCAGCGACGACGTTTCCGCCACCGAGTTCGATCTTGAGCANCCATCGTGAGC
GANGGNGTNCAGGTCGAGTAGTGACCGGGGCCGGAAGGACAACAACGNTGGAGTCGCCCCTTCGTCNAAANT
ACTTTTCTGGGNTCNGGGAAAAACGGGNGAACTCAAATTCTTTGCTTGCCCAGGGGNCCCCAGGCCCCAAGT
TGGAAATAAGGTTACTAAACTTTCACTCAACTGGGANGG

Sequences producing significant alignments: Score (bits) E-Value

gi|2463090|emb|CAA72965.1| diverged serine protease [Helico... 147 2e-34
gi|2463082|emb|CAA72961.1| diverged serine protease [Helico... 147 2e-34
gi|4760786|dbj|BAA77401.1| 30kP protease A (43k peptide) pr... 52 2e-05
gi|24583846|ref|NP_609551.1| CG16997-PA [Drosophila melanog... 51 3e-05
gi|31236527|ref|XP_319429.1| ENSANGP00000014448 [Anopheles ... 47 3e-04
gi|31217409|ref|XP_316419.1| ENSANGP00000021092 [Anopheles ... 47 6e-04
gi|12621934|gb|AAA18530.2| trypsin [Simulium vittatum] >gi|... 46 8e-04
gi|31243053|ref|XP_321961.1| ENSANGP00000013945 [Anopheles ... 45 0.002
gi|42524050|ref|NP_969430.1| trypsin [Bdellovibrio bacterio... 43 0.006
gi|50603930|gb|AAH77417.1| Unknown (protein for MGC:82165) ... 43 0.006

Frame = +2

```
Query: 8 MKGFAFVLLCALVAVQGRSTAVQSASRELATLGENPYVVHLRLAVSTSGLLKTCAASVIS
         MKGFAFVLLCALVAVQGR SR+ A LGE P+VVHLR+AV TSG L +C S+I
Sbjct: 1 MKGFAFVLLCALVAVQGRHRG--ELSRQAAALGEQPWVVHLRVAVETSGNLNSCVGSLID

Query: 188 PSWVLTAASCLEXNRYVWIRFGAVNVANPTLVFEAGNAGVRV---------LNNLGLYTA
             WVLTAASCL  +R++W+R+GAV+V  P+LV E  N+ +R+           N+GL +
Sbjct: 59  NQWVLTAASCLSGSRFIWVRYGAVDVIRPSLVTE--NSNIRIHPQYSWATGAFNVGLISI

Query: 341 NNRAVDFSQAA-TIAPVALSEAGVSDSGKLCVFGANSEGGPGELLSCATVGLEAQXDGXX
           N     F Q+   I+PV L    V DS   C +GA  +G PGE LSC     +E + G
Sbjct: 117 NR----FIQSTDNISPVPL-VGDVYDSAIFCGYGAREDGQPGEQLSCYPGVVEERDTGRL

Query: 518 XXXXXXXXATEFDLEXP 568
                  AT++D+  P
Sbjct: 172 VFNGEGAEATKYDIGAP 188
```

SK37

GGCNCGAGGAGAAGTAAGGTGACTGAAAANGCGTGTGATNGCNCTGTNGGNTNTTTGCCTAGCGGNTGNGGC
AGCCGCCCCCAAAGAATCCCAAAGGATTATNGGGGGATCTGTNACCAACATTGGCCAGTNCCNTATGATGGC
CTNACTCNTNTTNTTTNGGACCAACTNTGGCCACANACAGTCCTGCGGAGGCNCCATNATTAACAACCGANC
CGTCCTTACTGNTGNTNACTGCACCATNGGNGACCCAGTAAGCANATGGAGAGTTCGNGTNGGNTCCACAAA
CGCCAACGGNGGCGGNGTTGTTNACAACTCCCAGCAAATNATNAACCACCCNAGNTNCAACTNANGGACCTA
CNACANTGACGTTGCCATNATTCGNATTTCCGGCACATTNTCNTTNAACAACAACGNGCGCGCTG

Sequences producing significant alignments: Score (bits) E-Value

gi|2463084|emb|CAA72962.1|	trypsin-like protease [Helicover...	115	2e-25
gi|2463060|emb|CAA72950.1|	trypsin-like protease [Helicover...	114	9e-25
gi|2463056|emb|CAA72948.1|	trypsin-like protease [Helicover...	114	9e-25
gi|3355636|emb|CAA07611.1|	trypsin precursor [Lacanobia ole...	113	2e-24
gi|15072548|gb|AAK81696.1|	trypsin-like protein [Galleria m...	108	3e-23
gi|41057922|gb|AAR98918.1|	trypsin-like proteinase T25 prec...	107	6e-23
gi|6690630|gb|AAF24225.1|	trypsin-like PiT2b precursor [Plo...	105	4e-22
gi|3153855|gb|AAC36248.1|	trypsin [Plodia interpunctella] >...	105	4e-22
gi|6690632|gb|AAF24226.1|	trypsin-like PiT2b precursor [Plo...	104	5e-22
gi|2463058|emb|CAA72949.1|	trypsin-like protease [Helicover...	104	7e-22

```
Frame = +2
Query: 32  RVXALXXXCLAXXAAAPKESQRIXGGSVTNIGQXXMMAXLXXFXTNXGHXQSCGGXXINN
           R  AL   C A  AA P   QRI GGSVT I +   +A L        + QSCGG  +NN
Sbjct: 2   RFLALLALCFAAVAAVPSNPQRIVGGSVTTIDRYPTIAALLYSWNLSAYWQSCGGTILNN

Query: 212 RXVLTXXXCTXGDPVSXWRVRVGSTNANGGGVVXNSQQXXNHPXXNXXTYXXDVAXIRIS
           R +LT   CT GD  + WR+RVGST AN GGVV N      HP  N  T   D+A +R +
Sbjct: 62  RAILTAAHCTAGDANNRWRIRVGSTWANSGGVVHNLAANIIHPSYNSRTMDNDIAVLRSA

Query: 392 GTXSXNNNXRA 424
            T S NNN RA
Sbjct: 122 TTFSFNNNVRA 132
```

SK38

AGACCGGTGAACATCGAGGAGAGGTCGTGGAGGAATTTACCATCTAGCCACCATCGCCGTTCGCCAATTACA
CCNTGGNTGATACCGAAAAGGCGCCGNAGCCGCAGCCCCAACAGCAGCAGCANCAAGAGCAACANTCCCCAC
AGCAACCGCAACAAGCTAAAGCGGNTAAACAAAAGCAGGTCATTGCTGAGAAAGTTTCGGGAACCGTCAAAT
GGTTTAATGTGAAGAGTGGATATGGTTTCATCAACAGGAANTGACACCAAGGGAGGGTTTTNTTTGTACATN
AAACTGCANTCGCCCGGAACAACCCTCGCAAGGNTGTGCGCTCGGTCGGCGACGGGGAGGCGGTGGAGTTTG
CCGTGGTTGCCGGGGAGAAAGGCTTTGAAGCAGCCGGANTGACTGGTCCCGGCGGTGAGCCAGTTAAGGGCT
CGCCCTATGCAGNTGACAAACGCCGGGGNTTCCATNGCCAATNTTACCCCCGTCAAGGTGGCGGACCTGTNG
GNGAAAGGCNCTCCACCTAAAAGGTGGAATGGGACTTTNNGGGGCCCCCCCCCCCACCCAAGGGGGGTTTCC
CAAGGGGGGATNAAAGGTCAAGGAANGGAGCCCGGGNGCCCCCNCCCCAANCGCANNTTNNTTTTCCGGCCC
NAAATTTTTCCGNGGGNGGGACCNCCCCGNGGGNGGGGNGNTTCCCGGGGGCCTTNTTNNAATT

Sequences producing significant alignments:	Score (bits)	E-Value
gi|49532669|dbj|BAD26606.1| Y-box protein [Bombyx mori]	147	2e-34
gi|48134295|ref|XP_393344.1| similar to Y-box protein Ct-p4...	114	3e-24
gi|22901742|gb|AAN10050.1| Y-box protein Ct-p50 [Chironomus...	113	5e-24
gi|22901740|gb|AAN10049.1| Y-box protein Ct-p40 [Chironomus...	113	5e-24
gi|1175568|sp|P41824|YBFH_APLCA Y-box factor homolog (APY1)...	110	2e-23
gi|16769538|gb|AAL28988.1| LD37574p [Drosophila melanogaste...	107	2e-22
gi|2970679|gb|AAC06034.1| Y box protein [Drosophila silvest...	106	6e-22
gi|27348122|dbj|BAC45236.1| Y-box binding protein [Oryzias ...	105	9e-22
gi|1363073|pir||A55971 Y box-binding protein 1 - rabbit >gi...	104	2e-21
gi|27807361|ref|NP_777240.1| nuclease sensitive element bin...	104	2e-21

```
Frame = +3
Query: 183 VIAEKVSGTVKWFNVKSGYGFINRX*HQGRVXFVHXTAXARNNPRKXVRSVGDGEAVEFA
           VIAEKVSGTVKWFNVKSGYGFINR   +  V FVH TA ARNNPRK VRSVGDGEAVEFA
Sbjct: 33  VIAEKVSGTVKWFNVKSGYGFINRNDTKEDV-FVHQTAIARNNPRKAVRSVGDGEAVEFA

Query: 363 VVAGEKXXXXXXXXXXXXXXVKGSPYAXDKRRGFHXQXYPRQGGGPVGE 509
           VVAGEK              VKGSPYA DKRRG+H Q +PRQGGG  GE
Sbjct: 92  VVAGEKGFEAAGVTGPGGEPVKGSPYAADKRRGYHRQYFPRQGGGRGGE 140
```

SK41

```
GAAAATGGNGTNTGTGCAAACAATCGCNACTNTAGTCGNGAAGACCTTCAAAATTGTCCTGAACNTAGNGAT
CCTNGTTNTGNNCAGANCTGGTTACAANGGCNANTTCNTGGGGGTGGNAGGTCCTTGGAACCTCANTGAGGA
NAAGAACCCTGNCNCCGANATCGTGGCCTCCGGNGTAATTGTAGGNTACCTGATATACACACTCGCGCAAGN
CGTCANTTACTNGTTTGGCACTACTGAACACAANANAGCCNTGTCANAGATAGTGATNAACTTCGTGGGAGG
GTTCCTGTGGATCGCGGTGGGGGCTGTGGCGCTGCACTACTGGGGCGGATACCAGGGCGAGCACCAGTNCCA
GTTNGTGTTCGNTGANAAACAGGTGGGTCTGGNTGTCGGNGCGCTCTGCGTGATCAACGGCGCA
```

Sequences producing significant alignments: Score (bits) E-Value

gi|31205049|ref|XP_311473.1| ENSANGP00000013724 [Anopheles ... 152 2e-36
gi|15292385|gb|AAK93461.1| LP03829p [Drosophila melanogaste... 131 5e-30

```
Frame = +2
Query: 2   KMXXVQTIATXVXKTFKIVLNXXILVXXRXGYXGXFXGVXGPWNLXEXKNPXXXIVASGV
           KM   +TI +   K FK+V+N  +L+  R GY G F G+ G WNL E K+P   IVASGV
Sbjct: 26  KMVSAETIGSIFIKVFKVVINIVVLIIYRTGYGGDFLGIGGTWNLNEEKSPDAEIVASGV

Query: 182 IVGYLIYTLAQXVXYXFGTTEHXXAXSXIVXNFVGGFLWIAVGAVALHYWGGYQGEHQXQ
            VG++IYT  Q + + FGTT+H    S  + N VG F+W+AVG  ALHYW GY  EH  +
Sbjct: 86  FVGFIIYTGVQLLTFGFGTTKHKYELSDTIMNVVGTFMWVAVGGTALHYWHGYLAEHDFE

Query: 362 XVFXXKQVGLXVGALCVINGA 424
            +   +  GL +GALCVINGA
Sbjct: 146 NITSERTAGLALGALCVINGA 166
```

SK46

CCGAGGGAGATNTGCGTTCAGNGGCGTAGGATACAGACTTGGTCAAACATCANATGATCATGAGCCAATACC
GACTGGGAATNAACAGCAACAAACTGACCAGCCTCGNTCCGTGCGTNTGCNACTGTACCGCGAAGGTTTNAN
TGTGGATTCCGGGCCGTTGCGGCAGTACACANACCCCGATCACGCTGATTTCNTCAATTGTNTACGTAGGGG
CGANATCCCCCCCNAGCTGTCGGGGTNTGGCGGCAAGGTCCGCGTGTCGTTGGAGGACCGGCGCCACGAGGA
GTGTCCGCNACACATCGCCAAGCCGNAGCCNTTCGCCGGCAAGGGACATTTGNTCGGNAGCCCGACCCCACC
AACAGTGGGGGNTACAGTGGGTGTAGNTTCCACCCCGAGCGACCGCGACGCCAACNT

Sequences producing significant alignments:	Score (bits)	E-Value
gi|16197781|gb|AAL13489.1| GH01724p [Drosophila melanogaste...	62	3e-09
gi|26333065|dbj|BAC30250.1| unnamed protein product [Mus mu...	62	5e-09
gi|31210073|ref|XP_314003.1| ENSANGP00000009984 [Anopheles ...	62	5e-09
gi|47207192|emb|CAF89858.1| unnamed protein product [Tetrao...	60	1e-08
gi|50737833|ref|XP_419216.1| PREDICTED: similar to hypothet...	59	3e-08
gi|45829832|gb|AAH68189.1| Unknown (protein for IMAGE:69601...	55	4e-07
gi|42658955|ref|XP_059929.5| PREDICTED: similar to hypothet...	53	2e-06
gi|30268361|emb|CAD89972.1| hypothetical protein [Homo sapi...	53	2e-06
gi|30268216|emb|CAD89897.1| hypothetical protein [Homo sapi...	53	2e-06
gi|26328157|dbj|BAC27819.1| unnamed protein product [Mus mu...	52	4e-06

Frame = +2

```
Query: 14  AFXGVGYRLGQTSXDHEPIPTGNXQQQTDQPRSVRXXLYREGXXVDSGPLRQYTXPDHAD
           A  G G RLG T  DH  + T       +    V   L+ +G  +D G LR Y  P + +
Sbjct: 182 AVWGQGMRLGMTDNDHTAVGTKKPAATIENKPVVVLKLWSQGFSIDGGELRHYDDPQNKE

Query: 194 FXNCXRRGXIPPXLSGXGGKVRVSLEDRRHEECPXHIAKPXPFAGKG 334
           F     RG IP  L G    V V +ED RHE+ P        F G G
Sbjct: 242 FLETVMRGEIPQELLEMGRMVNVDVEDHRHEDFKRQ-PVPQTFKGSG 287
```

SK49

CTCGGATATTACACGTGGATTGTAATCCGTGACTAACCAAAAATGGGCAAGGAAAAGACTCACATTAACATT
GTCGTCATCGGACACGTCGACTCCGGCAAGTCCACAACCACTGGTCACTTGATCTACAAATGCGGTGGTATC
GACAAGCGTACCCATCGAGAAGTTCGAGAAGGGAGGGCCCAGGAAAATGGGTAAGGGGTTCCTTTAAAATAT
GCCTGGGGTATTTGGGACAAAACTGAAAGGCTGAAGCGTGAACGTGGGAATCACCATTCGANATTCGCCCTN
GGGNANGTTCGAGACCGCTAAATACTATGTCACCATCATCGACGCCCCTGGACACAGAGATTTCATCAAGAA
CATGATTACTGGAACCTCCCAGGCTGATTGCGCCGTGCTCATTGTCGCCGCCGGTACCGGTGAGTTCGAGGC
TGGTATCTTCAAGAACGGACAGACCCGTGAGCACGCTCTGCTCGCTTTCACCCTTGGTGTCAAACAGCTGAT
CGTTGGTGTCAACAAAATGGACTTCACTGAGCCTTCNTTCCAGGGAATNCCGTTTCGNGGGAATCAAGAAGG
AAGTNTTCTTTTTCATTNAANAAAATTGGNTACAAACCAAATTGCCGTNGTTTTTGTACCCCATTTNTGGAT
GGGACCGGGAAACAACANTGTTGGGAGCCCCTNTANCCAAAATGNCCCT

Sequences producing significant alignments: Score (bits) E-Value

gi|2190621|gb|AAC47584.1| elongation factor-1 alpha [Schizu... 171 1e-41
gi|6901563|gb|AAF31079.1| elongation factor-1 alpha [Simuli... 171 1e-41
gi|5052473|gb|AAD38565.1| elongation factor-1 alpha [Papaip... 170 2e-41
gi|51464172|ref|XP_496711.1| PREDICTED: similar to elongati... 155 6e-55
gi|82773|pir||A25938 translation elongation factor eEF-1 al... 139 2e-53
gi|33359649|gb|AAQ17072.1| translation elongation factor 2 ... 135 6e-51
gi|2668565|gb|AAB88586.1| translation elongation factor 1-a... 135 6e-51
gi|119147|sp|P18624|EF1A_DICDI Elongation factor 1-alpha (E... 141 6e-51
gi|7275|emb|CAA39443.1| elongation factor 1 alpha [Dictyost... 141 6e-51
gi|7277|emb|CAA39442.1| elongation factor 1 alpha [Dictyost... 141 6e-51
gi|50254805|gb|EAL17550.1| hypothetical protein CNBM1160 [C... 134 1e-50

```
Frame = +2
Query: 263 GITIRXSPXGXFETAKYYVTIIDAPGHRDFIKNMITGTSQADCAVLIVAAGTGEFEAGIF
           GITI +     FET KYY T+IDAPGHRDFIKNMITGTSQADCAVLIVA GTGEFEAGI
Sbjct: 54  GITIDIA-LWKFETPKYYCTVIDAPGHRDFIKNMITGTSQADCAVLIVAGGTGEFEAGIS

Query: 443 KNGQTREHALLAFTLGVKQLIVGVNKMDFTEPSFQGXPFRGNQEGSXLFHXXKLXTNQIA
           KNGQTREHALLAFTLGVKQLIVGVNKMD TEP    + F+   E +      K+  N  A
Sbjct: 113 KNGQTREHALLAFTLGVKQLIVGVNKMDSTEPPYSESRFK-EIEKEVSAYVKKVGYNPKA

Query: 623 VVFVP-HXWMG 652
           V FVP   W G
Sbjct: 172 VAFVPISGWHG 182
```

SK50

ATCTGGCACCACACCTTCTACAATGAGCTGCGTGTGGCCCCCGAGGAGCACCCCGTGCTGNTCACCGAGGCC
CCGNTCAACCCCAAGGCCAACAGAGAGAAGATGACACAGATCATGTTCGAGACGTTCAACACGCCCGCCATG
TACGTCGCCATCCAGGCCGTGCTCTCGCTGTACGCCTCCGGTCGTACCACCGGTATCGTGCTGGACTCCGGC
GACGGTGTNTCGCACACGGTGCCCATCTACGAGGGCTACGCGCTGCCGCACGCCATCCTGCGTNTGGACTTG
GCCGGCCGCGACCTCACAGACTACCTGATGAAGATCCTCACCGAGCGCGGNTACTCGTTCACCACCGCCGAG
CGTGAGATCGTGCGCGACATCAAGGAGAAGCTCTGCTACGTTGCGCTCGACTTCGAGC

Sequences producing significant alignments: Score (bits) E-Value

gi|14010639|gb|AAK52066.1| actin [Heliothis virescens] 273 1e-72
gi|46371991|gb|AAS90632.1| actin [Cydia pomonella] 272 2e-72
gi|6946691|emb|CAB72314.1| actin [Daphnia pulex] 272 2e-72
gi|48137684|ref|XP_393368.1| similar to Actin-5C [Apis mell... 272 2e-72
gi|21430178|gb|AAM50767.1| LD18090p [Drosophila melanogaste... 272 2e-72
gi|19528371|gb|AAL90300.1| RE02927p [Drosophila melanogaste... 272 2e-72
gi|19526213|gb|AAL89658.1| cytoplasmic actin A3a1 [Helicove... 272 2e-72
gi|46561734|gb|AAT01072.1| putative cytoplasmic actin A3a1 ... 272 2e-72
gi|1168323|sp|P45886|ACT3_BACDO Actin 3, muscle-specific >g... 272 2e-72
gi|728796|sp|P41339|ACTA_LIMPO Actin, acrosomal process iso... 272 2e-72

Frame = +1

Query: 1 IWHHTFYNELRVAPEEHPVLXTEAPXNPKANREKMTQIMFETFNTPAMYVAIQAVLSLYA
IWHHTFYNELRVAPEEHPVL TEAP NPKANREKMTQIMFETFN+PAMYVAIQAVLSLYA
Sbjct: 86 IWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKMTQIMFETFNSPAMYVAIQAVLSLYA

Query: 181 SGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRXDLAGRDLTDYLMKILTERGYSFTTAE
SGRTTGIVLDSGDGVSHTVPIYEGYALPHAILR DLAGRDLTDYLMKILTERGYSFTTAE
Sbjct: 146 SGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRLDLAGRDLTDYLMKILTERGYSFTTAE

Query: 361 REIVRDIKEKLCYVALDFE 417
EIVRDIKEKLCYVALDFE
Sbjct: 206 SEIVRDIKEKLCYVALDFE 224

SK53

TTTGGTCGACACTAGNGGATCCAAAGAATTCGGCATGAGGTTCACGCCACTCAGCAGATTTNTTAACCACCC
CAACTACAGCGGATGGNNNCTCNNNAATGACGTTGCCATCATTCGCGTTTCTTCCACATTCTCCTTCAACAA
CAACGTCGCTTCTGCTTCCATCGCCGGCTCCAACTACAACCTTGGTGACAACCAGGTCGTCTGGGCTACTGG
ATGGGGAACCACTTCTNCTGGNGGATCTCTCTTCTGAACAGCTCCGTCACGTGCAAATCTTGGACCGTNACC
AACTACTTTGCAGAACTCGCTACCAAACTCTTGGCAGGACCGCACTGACAACATGTTNGCTCCGGGTGGCTC
GACCAAGGGGGCGCGACCAGTGCCANGGAGACTCCGGGGGNCTTTTTACCCAACCGGGGGCGTTGTTGGGGC
TGCTCAANGGGGCAAAAATCCCCCTTGCTTTNTACCCAGGGGTNACCCTCGCGTTTTAAAAACCCATCTTGG
ATTAAAACAANGCTTAAGGGTTTNAAAAACCGGTNTTTAAAACANCCAATAAAAATTGATTTANGATGNAAA
AAAAAAAAAAAAA

Sequences producing significant alignments:	Score (bits)	E-Value
gi|2463070|emb|CAA72955.1| trypsin-like protease [Helicover...	83	3e-15
gi|3355636|emb|CAA07611.1| trypsin precursor [Lacanobia ole...	82	8e-15
gi|8347662|gb|AAF74745.1| trypsin precursor Hz11 [Helicover...	81	1e-14
gi|8347644|gb|AAF74736.1| trypsin precursor AiJ3 [Agrotis i...	79	5e-14
gi|298974|gb|AAB26023.1| alkaliphilic serine protease P-IIc...	61	1e-13
gi|2463060|emb|CAA72950.1| trypsin-like protease [Helicover...	78	1e-13
gi|2463056|emb|CAA72948.1| trypsin-like protease [Helicover...	78	1e-13
gi|6690630|gb|AAF24225.1| trypsin-like PiT2b precursor [Plo...	65	3e-13
gi|8347664|gb|AAF74746.1| trypsin precursor Hz42 [Helicover...	77	3e-13
gi|2463084|emb|CAA72962.1| trypsin-like protease [Helicover...	76	5e-13
gi|8347654|gb|AAF74741.1| putative trypsin precursor AiG8 [...	75	1e-12

```
Frame = +2
Query: 68  HPNYSGWXLXNDVAIIRVSSTFSFNNNVASASIAGSNYNLGDNQVVWAXXXXXXXXXXXXL
           HP ++ W L NDVA++RVS+TFSFNNNV +ASIAG+NYN+GDNQ VWA
Sbjct: 104 HPQFNRWNLNNDVAVLRVSNTFSFNNNVRAASIAGANYNVGDNQAVWAAGWGDTFYGSE-

Query: 248 F*TAPSRANLGPXPTTLQNSLPNSWQDR----TDNMXAPGGSTKGARPVPXRLRGXFYPT
                          + QN+  N++  R     +NM   G +  G         G  Y
Sbjct: 163 --QGSEQLRHVQLSIVNQNTCRNNYATRGVLVNENMICAGWPSGGRDQCQGDSGGPLYHN

Query: 416 GGVVGAAQXGKN 451
           G VVG    G N
Sbjct: 221 GIVVGVCSFGIN 232
```

SK54

CAATAATGTTCTCGCTCGGAAAGTGCGTTTTGGTCTTGTGCGCTGCTGTTGCAGCAAACGGGTTCTCCCTGG
GACCGACCGACATCATTTTCCACTTGTTCACAAGGTCGAACCCACAAGTCAGCCAGCCGATATTCCCCTCGA
TCAACTCCATCCTCGNTGCAAGTCTNTTCAATCCAGAGGTGAACACCGTCATCACCGTCCACAGCAATGGCG
AGGACGTTACTGGAAACTTCAACGCTTTCGTCGTTCTGGTCACTTACAGGCCGAGAATGTCAACGTTCTCGC
CGTGGACTGGAGCAAGGCTTCCTCCATGTACACCGAAGGCCTCGCCAACGCCGAGCAGGTCGGCAAGGTCAT
CGCAGACTTCATAAACATTTTGTCCCAGTCTTTCTCATACATGCCGAGTCAAGTGCG

Sequences producing significant alignments:	Score (bits)	E-Value
gi|50979160|ref|NP_001003319.1| lipase [Canis familiaris] >...	47	1e-07
gi|126316|sp|P06857|LIP1_CANFA Pancreatic lipase related pr...	47	5e-07
gi|3318843|pdb|1RP1| Dog Pancreatic Lipase Related Protein 1	47	5e-07
gi|163944|gb|AAA30840.1| pancreatic lipase precursor	47	5e-07
gi|31209547|ref|XP_313740.1| ENSANGP00000003833 [Anopheles ...	40	6e-05
gi|51874067|gb|AAH78528.1| Unknown (protein for MGC:85357) ...	44	7e-05
gi|27482986|ref|XP_058404.2| PREDICTED: similar to lipase, ...	45	9e-05
gi|14091772|ref|NP_114470.1| pancreatic lipase related prot...	47	1e-04
gi|5453920|ref|NP_006220.1| pancreatic lipase-related prote...	44	2e-04
gi|19343958|gb|AAH25784.1| Pancreatic lipase-related protei...	44	2e-04
gi|9256628|ref|NP_061362.1| pancreatic lipase related prote...	43	4e-04

```
Frame = +2
Query: 263 QAENVNVLAVDWSKAS-SMYTEGLANAEQVGKVIADFINILSQSFSYMPSQV 415
           + E VN + VDW K S + YT+   N   VG  +A  +++LS ++SY PSQV
Sbjct: 114 KVEEVNCICVDWKKGSQTSYTQAANNVRVVGAQVAQMLSMLSANYSYSPSQV 165

Frame = +3
Query: 90  FHLFTRSNPQVSQPIFPSINSILXASLFNPEVNTVITVH 206
           F L+T  NP   Q + PS  S + AS F  +  T  T+H
Sbjct: 56  FLLYTNKNPNNFQTLLPSDPSTIEASNFQTDKKTRFTIH 94
```

SK57

GTTCTCGGACGCCAGATTCCTAAATAGTTTTAGGAATTATTAAAAATGGGATTCGTGAAAGTCGTGAAGAAC
AAGCAATACTTTAAGAGGTACCAAGTTAAGTTCAAGAGGCGTCGTGAGGGAAAAACCGACTACTATGCCCGT
AAACGCCTTGTTGTTCAAGACAAGAACAAATACAACACACCCAAGTACCGCCTGATCGTCCGTCTGTCCAAC
AAGGATGTGACCTGCCAGGTGGCTTACTCCCGCATTGAGGGAGACCACATTGTCTGTGCTGCCTACTCCCAT
GAACTGCCGCGTTACGGCGTTAAAGTAGGTCTGACCAACTACGCAGCTGCCTACTGCACGGGGCTGCTGCTT
GCAAGAAGACTGCTCCAGAGACTTGGTCTGGACTCCTTATACAGTGGTGCAACAGAAGTCACTGGTGACGAA
TACAATGTTGAACCTGTAGACAACGGCCCGGGCGCTTTCAGATGTTACCTTGATGTGGGTCTAGCACGTACC
ACTACTGGTGCTCGTGTCTTTGGAGCCATGAAAGGTGCAGTTGATGGTGGCCTCAATGTCCCTCACTNCATC
AAGANGTTCCCTGGNTATGATGCCCGAAGCAAAGAAATTCAATGCAGAAGTACACAGACTCACATCTTTGGN
TTACACGTGGCTGAGTACTGCNC

Sequences producing significant alignments: Score (bits) E-Value

gi|3282245|gb|AAC24960.1| ribosomal protein L5 [Bombyx mori... 362 4e-99
gi|38047859|gb|AAR09832.1| similar to Drosophila melanogast... 338 8e-92
gi|30923541|gb|EAA46019.1| CG17489-PA.3 [Drosophila melanog... 338 8e-92
gi|2809379|gb|AAB97731.1| ribosomal protein L5 [Anopheles g... 337 1e-91
gi|31238477|ref|XP_319782.1| ENSANGP00000005182 [Anopheles ... 337 1e-91
gi|3153904|gb|AAC17448.1| RPL5A-related protein [Helianthus... 333 2e-90
gi|38048341|gb|AAR10073.1| similar to Drosophila melanogast... 331 7e-90
gi|27503217|gb|AAH42258.1| MGC53393 protein [Xenopus laevis] 320 1e-86
gi|45383023|ref|NP_989912.1| ribosomal protein L5 [Gallus g... 317 1e-85
gi|14591909|ref|NP_000960.2| ribosomal protein L5; 60S ribo... 317 2e-85

Frame = +1

```
Query: 46 MGFVKVVKNKQYFKRYQVKFKRRREGKTDYYARKRLVVQDKNKYNTPKYRLIVRLSNKDV
          MGFVKVVKNKQYFKRYQVKFKRRREGKTDYYARKRLVVQDKNKYNTPKYRLIVRLSNKDV
Sbjct: 1  MGFVKVVKNKQYFKRYQVKFKRRREGKTDYYARKRLVVQDKNKYNTPKYRLIVRLSNKDV

Query: 226 TCQVAYSRIEGDHIVCAAYSHELPRYGVKVGLTNYAAAYCTGXXXXXXXXXXXXXDSLYS
            TCQVAYSRIEGDHIVCAAYSHELPRYGVKVGLTNYAAAY TG             D+LY+
Sbjct: 61  TCQVAYSRIEGDHIVCAAYSHELPRYGVKVGLTNYAAAYSTGLLLARRLLQRLGLDTLYT

Query: 406 GATEVTGDEYNVEPVDNGPGAFRCYLDVGLARTTTGARVFGAMKGAVDGGLNVPHXIKXF
            G T+VTGDEYNVEPVDNGPGAFRCYLDVGLARTTTG RVFGAMKGAVDGGLNVPH IK F
Sbjct: 121 GTTDVTGDEYNVEPVDNGPGAFRCYLDVGLARTTTGRRVFGAMKGAVDGGLNVPHSIKRF

Query: 586 PGYDARSKEIQCRSTQTHIFGLHVAEY 666
            PGYDA SK+      + HIFGLHVAEY
Sbjct: 181 PGYDAESKKFNAEVHRAHIFGLHVAEY 207
```

SK58

GTTCTCGGACGCCAGATTCCTAAATAGTTTTAGGAATTATTAAAAATGGGATTCGTGAAAGTCGTGAAGAAC
AAGCAATACTTTAAGAGGTACCAAGTTAAGTTCAAGAGGCGTCGTGAGGGAAAAACCGACTACTATGCCCGT
AAACGCCTTGTTGTTCAAGACAAGAACAAATNCAACACACCCAAGTNCCGCCTGATCGTCCGTCTGTCCAAC
AAGGATGTGACCTGCCAGGTGGCTTACTCCCGCATTGAGGGAGACCACATTGTCTGTGCTGCCTACTCCCAT
GAACTGCCGCGTTACGGCGTTAAAGTAGGTCTGACCAACTACGCAGNTGCCTACTGCACGGGGCTGNTGCTT
GCAAGAAGACTGCTCCAGAGACTTGGTCTGGACTCCTTATACAGTGGTGCAACAGAA

Sequences producing significant alignments: Score (bits) E-Value

gi|3282245|gb|AAC24960.1| ribosomal protein L5 [Bombyx mori...	209	2e-53
gi|2809379|gb|AAB97731.1| ribosomal protein L5 [Anopheles g...	201	3e-51
gi|31238477|ref|XP_319782.1| ENSANGP00000005182 [Anopheles ...	200	7e-51
gi|3153904|gb|AAC17448.1| RPL5A-related protein [Helianthus...	199	2e-50
gi|30923541|gb|EAA46019.1| CG17489-PA.3 [Drosophila melanog...	198	3e-50
gi|30923542|gb|EAA46020.1| CG17489-PD.3 [Drosophila melanog...	198	3e-50
gi|42415363|gb|AAS15651.1| SD13191p [Drosophila melanogaster]	198	3e-50
gi|38048341|gb|AAR10073.1| similar to Drosophila melanogast...	198	3e-50
gi|38047859|gb|AAR09832.1| similar to Drosophila melanogast...	198	3e-50
gi|1374957|gb|AAC05598.1| ribosomal protein L5 [Styela clav...	192	2e-48

Frame = +1

```
Query: 46 MGFVKVVKNKQYFKRYQVKFKRRREGKTDYYARKRLVVQDKNKXNTPKXRLIVRLSNKDV
          MGFVKVVKNKQYFKRYQVKFKRRREGKTDYYARKRLVVQDKNK NTPK RLIVRLSNKDV
Sbjct: 1  MGFVKVVKNKQYFKRYQVKFKRRREGKTDYYARKRLVVQDKNKYNTPKYRLIVRLSNKDV

Query: 226 TCQVAYSRIEGDHIVCAAYSHELPRYGVKVGLTNYAXAYCTGXXXXXXXXXXXXXDSLYS
            TCQVAYSRIEGDHIVCAAYSHELPRYGVKVGLTNYA AY TG             D+LY+
Sbjct: 61  TCQVAYSRIEGDHIVCAAYSHELPRYGVKVGLTNYAAAYSTGLLLARRLLQRLGLDTLYT

Query: 406 GATE 417
            G T+
Sbjct: 121 GTTD 124
```

SK59

TTGAGCTACTGGGCAGAGGAGTACAATTTCAGTGAACGAGAGACCTTCCTGAACCAGTTCCCCCACTACAAG
ACTTACATACAGGGTCTGGATATCCACTTCATCAGGGTGAAGCCACAGGTACCACAAAATGTGGAGATTGTC
CACTTCTCTTAATGCACGGCTGGCCAGGGTCTGTGCGAGAGTTCTATGAAGCCATTCCTCTGCTCACCCGCC
AGCAACCAGGATACAACTTTGCTTTCGAAGTTATTGTACCAAGTATACCTGGATATGGATTTTCACAAGGAG
CCGTCCGCCCGGGGCTCGGAGCACCTCAAGTATCAGTGATCTTCAAGAACCTGATGAACCGGCTCGGGTACG
ACAAGTTCTACATTCAGGGAGGAGACTGGGGCGCAGTAATAGCGTCTACTATGGCTACTATATTCCCAGAAT
TGCTTCTTGGACATCATTCAAACATGCTGACGGTTCATAACAGCAAATCAACGTTGAAGATGTTCATCGGCG
CATATTTCCCGTCGTTCGTAATGCCCGAGCACTTGGTCGACAGACTGTACCCACTGTCCAGTTTGTTCGCTT
ACGTCATGGAAGAGTTCGGCTACATGCACCTGCAAGCCACTAAACCTGATACTATTGGTATACCTCTAACAG
ACTCCCCAGCTGGTCTCCTCGCATATATTTTAGAGAAATTCTCCACATGGACAAAGAGGAGCCACAAGTTCA
AAGAAGACGGTGGCCTTGAATTCAGGTTCACAAAAGACCAGCTCCTAGACAATCTAATGATCTACTGG

Sequences producing significant alignments: Score (bits) E-Value

gi|2661096|gb|AAB88192.1| epoxide hydrolase [Trichoplusia ni] 296 7e-98
gi|36957250|gb|AAQ87024.1| juvenile hormone epoxide hydrola... 296 4e-96
gi|1658003|gb|AAB18243.1| microsomal epoxide hydrolase [Tri... 289 2e-94
gi|48099641|ref|XP_394922.1| similar to juvenile hormone ep... 278 1e-85
gi|20502972|gb|AAM22694.1| juvenile hormone epoxide hydrola... 269 4e-85
gi|20502974|gb|AAM22695.1| juvenile hormone epoxide hydrola... 265 2e-83
gi|31211627|ref|XP_314783.1| ENSANGP00000014385 [Anopheles ... 250 2e-77
gi|12585256|sp|Q25489|HYEP_MANSE Juvenile hormone-specific ... 235 3e-76
gi|39104464|dbj|BAD04047.1| microsomal epoxide hydrolase [D... 254 4e-74
gi|17944457|gb|AAL48118.1| RH03206p [Drosophila melanogaster] 254 2e-73

Frame = +3

```
Query: 144 PLLLMHGWPGSVREFYEAIPLLTRQQPGYNFAFEVIVPSIPGYGFSQGAVRPGLGAPQVS
           PLLL+HGWPGSVREFYEAIPLLT      +FAFEVIVPS+PGYGFS  AVRPGLGAPQ+
Sbjct: 146 PLLLLHGWPGSVREFYEAIPLLTAVSKDRDFAFEVIVPSLPGYGFSDPAVRPGLGAPQIG

Query: 324 VIFKNLMNRLGYDKFYIQGGDWGAVIASTMATIFPELLLGHHSNMLTVHNSKSTLKMFIG
           V+ KNLM+RLGY +FY+QGGDWGA+I + + T+FP+ +LG+H+NM  V ++KSTL   +G
Sbjct: 206 VVMKNLMSRLGYKQFYLQGGDWGALIGNCIVTLFPKDILGYHTNMPIVMSAKSTLFELLG
Query: 504 AYFPSFVMPE-HLVDRLYPLSSLFAYVMEEFGYMHLQATKPDTIGIPLTDSPAGLLAYIL
           + FPS ++ +    +RLYPLS+ FA ++ E GYMH+Q+TKPDT+G+ L+DSPAGLLAYIL
Sbjct: 266 SVFPSLILEDMSTYERLYPLSTRFANLLRETGYMHIQSTKPDTVGVALSDSPAGLLAYIL

Query: 681 EKFSTWTKRSHKFKEDGGLEFRFTKDQLLDNLMIYW 788
           EKF+TWT+     K +GGL++RFT+DQL+DNLM+YW
Sbjct: 326 EKFATWTRPDLMSKPNGGLDYRFTRDQLIDNLMMYW 361
```

SK63

GAGATCAAAACAAAGAGGAATACGCATACGTTTTTATTCTAATAATTACCCTATATTATGCTAAACAGGATA
TCAATAGTTGCCGATGGCTGAAGAAAAGGATTTTAACGATCTCTTGGATGATATTTTCCTTGGTGAAGAGAA
GCAGCACAAGGAAAGTTATGACCAAGGTTTCAAAGCTGGTAGTGAAGCGGGGAATCCTGAAGGCTATCACCT
CGGNTACCATAAAGGAGCANAATTAGGGCGGGAACTTNNTTTCTATTTTGGCACANTCTCACACCATATAAA
CGAAAACAANTNGCCTGNGAAANACNCTGAGAAGTTGTTAACTCAACNGACCAAAGTAAAGGAGCTGATTGA
GGCTTTTCCAAGGACTAATTCCCNGGAACCTGATGTACTTACCATGGGCNAGGNGTGTNCCGGGTNANGATA
AAAANAACNGGCCCTNCTNAANATATTCTNGGNGGATCATNTNATGCAAGAATCTCATTTTGATTTTGGTGA
CCCACAATNACTTTACAATAACCNANATTCGTATAAAATAGACTNCGTTATGAAGGCTTTGACACCCCTCCT
GGGCCTGNGTAATGTTTNTTGNGGGGNGGATACACAAAAAACCNNGGTTNTTTTTTTGNCCCNCCCCNNGGG
GAAANTTTNGGGGAAG

Sequences producing significant alignments: Score (bits) E-Value

gi|28573370|ref|NP_788330.1| CG33154-PA [Drosophila melanog... 78 1e-13
gi|50747992|ref|XP_421070.1| PREDICTED: similar to oral can... 48 2e-04
gi|46101056|gb|EAK86289.1| hypothetical protein UM04834.1 [... 48 2e-04
gi|50554011|ref|XP_504414.1| hypothetical protein [Yarrowia... 42 0.009
gi|42761493|gb|AAS45311.1| hypothetical protein [Dictyostel... 40 0.043

Frame = +2

```
Query: 98  KDFNDLLDDIFLGEEKQHKESYDQGFKAGSEAGNPEGYHLGYHKGAXLGRELXFYFGTXS
           +D NDL DDI L EEK+ +  Y++G K G E GN EGY LGY +G  LG EL    G
Sbjct: 6   RDINDLFDDIVLTEEKEARLGYEEGLKDGQEQGNEEGYKLGYAQGVSLGEELGKILGQVV

Query: 278 HHINENXXPXKXXEKLLTQXTKVKELIEAFPRTNSXEPDVL 400
                     K  +K+     +++ LIE FPRTN  + D++
Sbjct: 66  AQ-----QQLKHTDKVRRSLEQLRSLIEEFPRTNDPQADIV 101
```

SK65

ACAACGATGATGAAGGCAACTTTGACTTGAAGAACACCTTGGATGACGGTAAGATTGCTGATGATACCAAAG
CACAGAAGGACAGTTTCCTGGGCAACGATATCGTTGACGATGACAAGGACGAAGGTGATGATGATGATGACT
CCGCTGACGAAGATGAAGATGATGAAGACGAANATGACGACNCCAGNGAGGACTACNACGGTGACGATGAAG
TGGACCNCNAAGTAAAACTTATCGAGAAACCTTCAACCACTACTGNGGACTACGCCGCAGNACTAAATCATC
TGNTNTTCCANANGAAGAAACNGACNTCAAACTATTTGAACACCACNTTACCAATGNTGAGTCATNAAGTAA
CNTNCCNTNCCAGAGGCTATTNAGGCTGNAGAGGAACACNGCCNCTTCCGTTACNTGTGGGCAAGGTTNCAT
CGGNATCTTTTGNGCTCTGNTGGTGGCATNGTNATTCCAAAGGGGGAGGCTCTGTGCCCTTAAAAAACAAGG
GGGATCACATCCNCNGTGCCCTTTTGCTGCCTGAGCCAGTTCCGGCGACAAAAAAGGCTGNGGGNTGGNGGN
CNCCCGGTNTCTNTGGACCCGGCAAGCCCGCCCCCCCCAANTTAAACGTTAACNCCGTTGNCACAGTAACGT
NAAAGCT

Sequences producing significant alignments:	Score (bits)	E-Value
gi|2564052|dbj|AB007832.1| Bombyx mori mRNA for Bm tracheal..	64	4e-07
gi|49529273|emb|CR543861.1| Acinetobacter sp. ADP1 complete...	46	0.099
gi|40994810|emb|BX323808.7| Zebrafish DNA sequence from clo...	40	6.1
gi|12718447|emb|AL513445.1|NCB7A16 Neurospora crassa DNA li...	40	6.1

```
Strand = Plus / Plus
Query: 27   ttgaagaacaccttggatgacggtaagattgctgatgataccaa 70
            |||||||||||||| |||||||||| ||| ||||||||||||
Sbjct: 3929 ttgaagaacaccttagatgacggtaaaattcctgatgataccaa 3972
```

SK70

GAAACATATTTTTATTGTGTTTAGTTTCTATATCCTGTGCTCTTGGAGAGCAGAGGATAGCGGGGGGTGCAC
TCACGACCATCAGCCAGTACCCGTTTGCGGCCGCCCTCCTCACCAACAGGGCTGGTGGTGACTACGTGCAGG
CGTGCGGCGGTACCATCATCACTCAGTCCGCCATCTTGTCTGCTGCTTCCTGTTTCTACACTGGCACTGCAC
AAAANTCCNCGGCAGCATGGGGCGCGCGNGTGGGTTCCTCTTACCGCAACTCCCAAGGNACCATTTACATAT
AANCANCNGTTANCNTTCACNANGGGTTTTTCCCCACTNCTTTNGNNAANNANNTTGGANNACTNCGCACCA
CCNGGACCATTACCTNCAGTGCTTATGTACANCCCGCCAGGATCGCTNGAGCTGGTTACAATNCCGGNGACA
ATNAANAAATTGGGCTTTTGGATGGGGGGCCNTATCTAACCNAGTGTNAGCATNAGAAAAACTGNGTNACGT
TCAAATTGGANAAATTGGGNAGACCTTCTGAACGGTACGCCCAGACCCCACAAACATTAACGNCACNTGACT
NCCTGGGATTTTTGGGGTTGGNCTCAGGGGACAAAGACCGGANAANTNGGGGACCNTTNTTGGNCCCCCTTG
GGAAAAAGANN

Sequences producing significant alignments:		Score (bits)	E-Value
gi|532085|gb|AAA84423.1|	trypsin	86	8e-18
gi|464957|sp|P35045|TRYA_MANSE	Trypsin, alkaline A precurso...	85	3e-17
gi|464962|sp|P35042|TRYP_CHOFU	Trypsin CFT-1 precursor >gi|...	84	3e-17
gi|464960|sp|P35047|TRYC_MANSE	Trypsin, alkaline C precursor	83	1e-16
gi|293226|gb|AAA29341.1|	trypsin	83	1e-16
gi|2463060|emb|CAA72950.1|	trypsin-like protease [Helicover...	81	2e-16
gi|2463056|emb|CAA72948.1|	trypsin-like protease [Helicover...	81	2e-16
gi|15072548|gb|AAK81696.1|	trypsin-like protein [Galleria m...	84	7e-16
gi|2463084|emb|CAA72962.1|	trypsin-like protease [Helicover...	80	2e-15
gi|464959|sp|P35046|TRYB_MANSE	Trypsin, alkaline B precurso...	82	2e-15

Frame = +3

```
Query: 15 LCLVSISCALGE--QRIAGGALTTISQYPFAAALLTNRAGGDYVQACGGTIITQSAILSA
          LCLVS++ AL E  QRI GG++TTI Q+P  +ALL +     Y QACGG I+   +ILSA
Sbjct: 10 LCLVSVA-ALPEKQQRIVGGSVTTIEQWPSGSALLYSWNLVTYSQACGGAILNTRSILSA

Query: 189 ASCFYTGTAQXSXAAWGARVGSSYRNSQGTIYI*XXVXXHXGFFPTXXXXXXGXLRTTXT
           A CF    A      W  R GS++ NS G ++    +  H  +           LR+  T
Sbjct: 69  AHCFIGDAAN----RWRIRTGSTWANSGGVVHNTALIIIHPSYNTRTLDNDIAILRSATT
Query: 369 ITXSAYVXPARIAXAGYN 422
           I  +    PA IA A YN
Sbjct: 125 IAQNNQARPASIAGANYN 142
```

SK72

GGTGACTGAAAATGCGTGTGATTGCACTGTTGGCTCTTTGCCTAGCGGCTGTGGCAGCCGCCCCCAAAGAAT
CCCAAAGGATTATTGGGGGATCTGTCACCAACATTGGCCAGTACCCTATGATGGCCTCACTCCTCTTCTCTT
GGACCAACTCTGGCCACAGACAGTCCTGCGGAGGCACCATCATTAACAACCGAGCCNTGCTTNTTNTNGTNC
NCNGTNCCCNATGGGGANCCCATTAGGCNAAGGNAAAGTCCTNGTGGGTTCNCCAANCNCCANNGGGGGNGG
GGTNGTTANNACCTCCNAGNAAATAATAAACCCCCCNGTTTCAACTCAGGGACNTCGAAAGTGACGTGGCCA
TAATTCGTTTTCCGGCACATTTCTTTTTAAAAAAACNGGCGCCTGTTTNNTNCCCGCTCAANTAANCNTTTN
GANAAANAAAGTCCTGNGNTNTTCGGNGGGGNGGANAANTNGGGGGGGGGGAAAAATTAGNACCCTTTTTNN
TGTNGAAATGNGTGTGGANACAAANATTTGCCNCGCTCCNCAGATTTGGGGGCTCANCCCCACAANATGTGG
TCCCCGGGNNGTGNAANGGCCCCNCNCCCCCGGGGGGGAAGGNGGGGNTTTTTTTTTTTN

Sequences producing significant alignments:	Score (bits)	E-Value
gi|2463060|emb|CAA72950.1| trypsin-like protease [Helicover...	69	6e-11
gi|2463056|emb|CAA72948.1| trypsin-like protease [Helicover...	69	6e-11
gi|2463084|emb|CAA72962.1| trypsin-like protease [Helicover...	68	1e-10
gi|15072548|gb|AAK81696.1| trypsin-like protein [Galleria m...	67	3e-10
gi|2463058|emb|CAA72949.1| trypsin-like protease [Helicover...	67	3e-10
gi|3355636|emb|CAA07611.1| trypsin precursor [Lacanobia ole...	65	9e-10
gi|464962|sp|P35042|TRYP CHOFU Trypsin CFT-1 precursor >gi|...	56	7e-07
gi|532085|gb|AAA84423.1| trypsin	56	7e-07
gi|464960|sp|P35047|TRYC MANSE Trypsin, alkaline C precursor	55	1e-06
gi|293226|gb|AAA29341.1| trypsin	55	1e-06

```
Frame = +3
Query: 12  MRVIXXXXXXXXXXXXXPKESQRIIGGSVTNIGQYPMMASLLFSWTNSGHRQSCGGTIIN
           MR++              P   QRI+GGSVT I QYP +A+LL+SW  S + Q+CGGTI+N
Sbjct: 1   MRILALVALCFAAVAAVPSNPQRIVGGSVTTIDQYPTIAALLYSWNLSTYWQACGGTILN

Query: 192 NRAXL 206
           NRA L
Sbjct: 61  NRAIL 65
```

SK76

ATCAAATATTATTTTATAATGACTTGTGCCAGGAGTGGTTCCACTTAGAGTAATGTAACTATAATGTTATTA
ACTCGGTCAGTCACATTATCTAAAAATATGAAGTGTGAATGTCAATAAGTTTATGTGATGGCTAATGTTTGA
CAGGTTAACATTTTCTATAGGTCACAGGGCTTTTTACCTGCTCATATTGGCTGTCAAATGAAAGACTGGGTC
TTTGTAACCTAAGAAGCCCTTATCCCTGCTAATATTATAAATGCGAAAGTAACTCTGTCTGTCTGTCTGTCT
GTTACGCTTTCACGTCTAAACCACTGAACCGATTTACAGAGATAGAGTTAACCTTGAGAAAGAACATAGGAT
AGTTTTTATCCCGGACTTTTGAAGAGGTCTCTTGAAAACGCGATATAACCGAGAAAAGCTAGTATTATATAT
TGTTAAACAATAGCCATACATAACAAACAGGGTGAAACATAGAGGTGCAACAAAATTTTTAAATGTTGCTCA
GATGAAAAATGTTCTTTACATTGCAAATTCGAGTTGGAAAATTAACTAATGCTTTGTTATAGGAAATGCAGC
CTGTAAACCAGAGATTATTTAATTCAAAGACTGACTATTTTTTTTTTACNGGTACCNCAAATTNCCTTGGCC
NGGTTTGGANGG

Sequences producing significant alignments: Score (bits) E-Value

gi|17979860|gb|AF330630.1|AF330630	Helicoverpa armigera mic...	92	2e-15
gi|14009480|gb|AF254789.1|	Autographa californica nucleopol...	88	3e-14
gi|51512139|gb|AY696174.1|	Plutella xylostella clone DBM01 ...	84	5e-13
gi|51242236|gb|AY603692.1|	Utetheisa ornatrix microsatellit...	76	1e-10
gi|32188040|dbj|AB112959.2|	Plutella xylostella CYP9G2 gene...	66	1e-07
gi|2282471|dbj|AB005911.1|	Bombyx mori DNA for xanthine deh...	60	7e-06

Strand = Plus / Plus

```
Query: 245 ctaatattataaatgcgaaagtaactctgtctgtctgtctgtctgt 290
           ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 112 ctaatattataaatgcgaaagtaactctgtctgtctgtctgtctgt 157

Query: 270 tctgtctgtctgtctgtctgt 290
           ||||||||||||||||||||
Sbjct: 169 tctgtctgtctgtctgtctgt 189

Query: 270 tctgtctgtctgtctgtctgt 290
           ||||||||||||||||||||
Sbjct: 165 tctgtctgtctgtctgtctgt 185
```

SK78

AGGAGACTCATCGNCATTNTTTTNAATTAAAGTTAATTTATCTGANGNGGGGTTTTTNCGCACCNTGGCTTN
GCCACGAGTTTTCTTTGACGTTTCTGCTGATGGTTCAGCTTTGGGAAGAATTGTTGTTGAGCTGAGAACAGA
TGTCACCCCCAAGACCTGTGAAAACTTCCGCGCCCTGTGCACCGGCGAGAAAGGTTTCGGCTACAAGAACTC
GACCTTCCCCGGGTGATCCCCAACTTCATGTTGCAAGGAGGTGACTTCACAAACCACAATGGCACTGGNGGN
ANGTCCNTCTACGGNGAGAAGTTTGCTGACGAGAACTTCGTCCTCAAACCCNCCGGCCCTGGAGTCCTGTCC
ATGGNCNACGCTGGCCCCCACACCAATGGATCCAGNTTTTCATCCCNCCCNGAAGACTTNTTGGGTGGNCGG
NAGACACGTCGTNTTCNGAACCCGTTGGNGAAGGNGTGGGTGTTGGCAAACCAGTTTAAACCTTCGNTTCCA
GTCTGGGGNGGCCTCANGNAAATCATTTGCTTCCGGTGGGGGCAACCCTTNT

Sequences producing significant alignments: Score (bits) E-Value

gi|2655159|gb|AAB87889.1|	cyclophilin 1 [Drosophila subobsc...	94	6e-39
gi|2655157|gb|AAB87888.1|	cyclophilin 1 [Drosophila pseudoo...	94	1e-38
gi|47117835|sp|P25007|CYPH_DROME	Peptidyl-prolyl cis-trans ...	92	4e-38
gi|33589296|gb|AAQ22415.1|	SD01793p [Drosophila melanogaste...	92	8e-38
gi|1703696|gb|AAB37708.1|	cyclophilin [Hemicentrotus pulche...	92	8e-38
gi|38048291|gb|AAR10048.1|	similar to Drosophila melanogast...	92	1e-37
gi|37362270|gb|AAQ91263.1|	peptidylprolyl isomerase A [Dani...	92	3e-37
gi|41152400|ref|NP_956251.1|	Unknown (protein for MGC:73102...	92	3e-37
gi|16768504|gb|AAL28471.1|	GM06533p [Drosophila melanogaste...	90	2e-36
gi|38541767|gb|AAH62863.1|	Wu:fb13h02 protein [Danio rerio]	93	3e-36

Frame = +1

```
Query: 226 RVIPNFMLQGGDFTNHNGTGGXSXYGEKFADENFVLKPXGPGVLSMXXAGPHTNGSXF
           RVIPNFM QGGDFTNHNGTGG S YG KFADENF LK  G G+LSM  AG +TNGS F
Sbjct: 56  RVIPNFMCQGGDFTNHNGTGGKSIYGNKFADENFQLKHTGTGILSMANAGANTNGSQF

Frame = +2
Query: 74  PRVFFDVSADGSALGRIVVELRTDVTPKTCENFRALCTGEKGFGYKNSTFPG*SPTSCCK
           PRVFFD++ADG  LGRI++ELR+DV PKT ENFRALCTGEKGFGYK S F    P   C+
Sbjct: 5   PRVFFDMTADGEPLGRIIMELRSDVVPKTAENFRALCTGEKGFGYKGSIFHRVIPNFMCQ
```

SK79

GTGAGAAGTAAGGTGACTGAAAATGCGTGTGATTGCACTGTTGGCTCTTTGCCTAGCGGCTGTGGCAGCCGC
CCCCAAAGAATCCCAAAGGATTATTGGGGGATCTGTCACCAACATTGGCCAGTACCCTATGATGGCCTCACT
CCTCTTCTCTTGGACCAGCTCTGGCCACAGACAGTCCTGCGGAGGCACCATCATTAACAACCGAGCCGGNCT
TANTGNTGNTTACTGCACCTTTGGTGACCCAGTAAGCAGATGGAGAGTTCGTGTCGGCTCCACAAACGCCAA
CGGTGGCGGTGTTGTTCACAACTTCCCAGCAAATTATCAACCACCCGAGCTACAACTCGAGGACTGTGGGAC
AATGACGTTGCCATCATTCGCATTTCCGGNACATTCTTCTTTAACAACAACGTGCGCGCTGGTTTCATNGNC
GCTTCCACTACAACCTTGGNGACAATAAGGCCGCTNGGCTACCGGTGGGGGAAGAATTTCNAAGGGGGTTAA
GGTTTTAACAAGTTTTGTACCTGCCAAATTTGGATGGGGACCAAAACATTTGCAACNCCNCTTCCCAANAAN
TTGGGGGGCCCTACCCCTTANNNNTTGGGTCNCCTGGGTCCATTTGGGGGGGGGACAAAGCCAAGANAANTC
CGGNGTCTTT

Sequences producing significant alignments: Score (bits) E-Value

gi|2463084|emb|CAA72962.1|	trypsin-like protease [Helicover...	114	2e-29
gi|2463060|emb|CAA72950.1|	trypsin-like protease [Helicover...	114	1e-28
gi|2463056|emb|CAA72948.1|	trypsin-like protease [Helicover...	113	2e-28
gi|3355636|emb|CAA07611.1|	trypsin precursor [Lacanobia ole...	112	1e-27
gi|6690630|gb|AAF24225.1|	trypsin-like PiT2b precursor [Plo...	103	1e-26
gi|6690632|gb|AAF24226.1|	trypsin-like PiT2b precursor [Plo...	103	1e-26
gi|41057922|gb|AAR98918.1|	trypsin-like proteinase T25 prec...	115	6e-26
gi|15072548|gb|AAK81696.1|	trypsin-like protein [Galleria m...	112	2e-25
gi|2463058|emb|CAA72949.1|	trypsin-like protease [Helicover...	100	1e-24
gi|7248888|gb|AAF43708.1|	trypsin-like protein precursor [H...	107	2e-22

```
Frame = +2
Query: 23  MRVIXXXXXXXXXXXXXPKESQRIIGGSVTNIGQYPMMASLLFSWTSSGHRQSCGGTIIN
           MR +              P   QRI+GGSVT I +YP +A+LL+SW  S + QSCGGTI+N
Sbjct: 1   MRFLALLALCFAAVAAVPSNPQRIVGGSVTTIDRYPTIAALLYSWNLSAYWQSCGGTILN

Query: 203 NRAGLXXXYCTFGDPVSRWRVRVGSTNANGGGVVHNFPANYQPP 334
           NRA L   +CT GD  +RWR+RVGST AN GGVVHN  AN   P
Sbjct: 61  NRAILTAAHCTAGDANNRWRIRVGSTWANSGGVVHNLAANIIHP 104

Frame = +1
Query: 358 DNDVAIIRISGTFFFNNNVRA 420
           DND+A++R + TF FNNNVRA
Sbjct: 112 DNDIAVLRSATTFSFNNNVRA 132

Frame = +3
Query: 324 INHPSYNSRTV 356
           I HPSYNSRT+
Sbjct: 101 IIHPSYNSRTM 111
```

SK80

TCAAGAAGGAAGTCTCCTCTTACATCAAGAAAATCGGTTACAACCCAGCTGCCGNCGCTTTCGTACCCATTT
CTGGATGGCACGGAGACAACATGTTGGAGCCCTCTACCAAAATGCCCTGGTTNAAGGGATGGTTGGTTGAGC
GCAAGGAGGGTAAGGCTGAAGGCAAGTGCCTTATTGAGGCTCTTGATGCCATCCTGCCCCCTGCTCGCCCCA
CAGACAAACCCCTGCGTCTTCCCCTTCAGGACGTATACAAAATCGGCGGTATTGGTACGGTGCCCGTAGGCA
GAGTCGAAACTGGTATCCTCAAGCCTGGTACCATCGTCGTCTTCGCCCCGCCAACATCACCACTGAAGTTAA
GTCCGTGGAGATGCACCACGAAGCCCTCCAGGAGGCTTGTACCCGGAGACAACGTTGGTTTCAACGTCAAAG
AACGTCTCCGTCAAGGAATTGCGTCGTGGNTACGTTGCTGGTGACTCCAAGAACAACCACCCAAGGTGCCGC
CGACTTCACTGNTTNAGGTCATCGTACTCAACCACCCCGGCNAATCTAAACGGATCACACCCGTCCTCGATT
GCACACANTTACAATTGCTTGCAAGTTCNCCGAAATNAAAAANAAAGTCAACCGNCNNCAGGTAATCCNCCG
AACCCACCCCAATTCCT

Sequences producing significant alignments: Score (bits) E-Value

gi|2688890|gb|AAC47892.1|	elongation factor-1 alpha [Attacu...	270	3e-71
gi|20302639|gb|AAM18815.1|	elongation factor-1 alpha [Cirin...	270	3e-71
gi|20302625|gb|AAM18808.1|	elongation factor-1 alpha [Attac...	270	3e-71
gi|20302621|gb|AAM18806.1|	elongation factor-1 alpha [Attac...	270	3e-71
gi|12836939|gb|AAK08676.1|	elongation factor-1 alpha [Dolba...	270	3e-71
gi|12836933|gb|AAK08673.1|	elongation factor-1 alpha [Sphec...	270	3e-71
gi|12963130|gb|AAK11159.1|	elongation factor-1 alpha [Phyll...	270	3e-71
gi|2688902|gb|AAC47898.1|	elongation factor-1 alpha [Coscin...	270	3e-71
gi|767869|gb|AAA93220.1|	elongation factor 1-alpha	270	3e-71

```
Frame = +3
Query: 3   KKEVSSYIKKIGYNPAAXAFVPISGWHGDNMLEPSTKMPWXKGWLVERKEGKAEGKCLIE
           KKEVSSYIKKIGYNPAA AFVPISGWHGDNMLEPSTKMPW KGWLVERKEGKAEGKCLIE
Sbjct: 157 KKEVSSYIKKIGYNPAAVAFVPISGWHGDNMLEPSTKMPWFKGWLVERKEGKAEGKCLIE

Query: 183 ALDAILPPARPTDKPLRLPLQDVYKIGGIGTVPVGRVETGILKPGTIVVFAPPTSPLKLS
           ALDAILPPARPTDKPLRLPLQDVYKIGGIGTVPVGRVETGILKPGTIVVFAP     ++
Sbjct: 217 ALDAILPPARPTDKPLRLPLQDVYKIGGIGTVPVGRVETGILKPGTIVVFAPANITTEVK

Query: 363 PWRCTTKPSRRLVPGDNVGFNVK----ERLRQGIASWXRCW*LQEQPPKVPPTSLXXVIV
                 +  +  VPGDNVGFNVK    + LR+G  +       +  PPK        VIV
Sbjct: 277 SVEMHHEALQEAVPGDNVGFNVKNVSVKELRRGYVAGDS----KNNPPKGAADFTAQVIV

Query: 531 LNHPG-XSKRITPVLDC 578
           LNHPG  S   TPVLDC
Sbjct: 333 LNHPGQISNGYTPVLDC 349
```

SK81

TCAAGAAGGAAGTCTCCTCTTACATCAAGAAAATCGGTTACAACCCAGCTGCCGTCGCTTTCGTACCCATTT
CTGGATGGCACGGAGACAACATGTTGGAGCCCTCTACCAAAATGCCCTGGTTCAAGGGATGGTTGGTTGAGC
GCAAGGAGGGTAAGGCTGAAGGCAAGTGCCTTATTGAGGCTCTTGATGCCATCCTGCCCCCTGCTCGCCCCA
CAGACAAACCCCTGCGTCTTNCCCTTTNAGGACGTATNCAAAATCGGGCGGTATTGGTACGGTGCCCNTNNG
GCNNAGTCAAAACTGGTATCCTCAAGCCTGGTACCCATCGTCGTCTTCGCCCCCGCCAACATCACCACTGAA
GTTAAGTCCGTGGAGATGCACCACGAANCCCTCCAGGAGGCTGTCCCGGAGACAACGTTGGTTTCAACGTCA
AGAACGTCTCCGTNAAGGAATTGCGTCGNGGTTACGTTGNTGGTGACTCCAANACAACCCACCCAAGGGTGC
CNGCCGACTTTCCTTGCTCAGGNNATCGTACTCAACCCCCCGGNCAAATNTNAAACGGATNCCNACCCGTNC
TNAATTGCCACAAAGTTNAATTTCCTGAAGTTCCCCAAATNAAAGAAAAGTNNNCCCTTTNCCGGGNAATCC
CCCGAACCCCCCCCAATNT

Sequences producing significant alignments: Score (bits) E-Value

gi|2688890|gb|AAC47892.1| elongation factor-1 alpha [Attacu...243 3e-63
gi|21624220|dbj|BAC01096.1| elongation factor 1a [Cepora iu...243 3e-63
gi|20302639|gb|AAM18815.1| elongation factor-1 alpha [Cirin...243 3e-63
gi|20302625|gb|AAM18808.1| elongation factor-1 alpha [Attac...243 3e-63
gi|20302621|gb|AAM18806.1| elongation factor-1 alpha [Attac...243 3e-63
gi|12836939|gb|AAK08676.1| elongation factor-1 alpha [Dolba...243 3e-63
gi|12836933|gb|AAK08673.1| elongation factor-1 alpha [Sphec...243 3e-63
gi|12963130|gb|AAK11159.1| elongation factor-1 alpha [Phyll...243 3e-63
gi|2688902|gb|AAC47898.1| elongation factor-1 alpha [Coscin...243 3e-63
gi|767869|gb|AAA93220.1| elongation factor 1-alpha 243 3e-63

Frame = +3

```
Query: 3   KKEVSSYIKKIGYNPAAVAFVPISGWHGDNMLEPSTKMPWFKGWLVERKEGKAEGKCLIE
           KKEVSSYIKKIGYNPAAVAFVPISGWHGDNMLEPSTKMPWFKGWLVERKEGKAEGKCLIE
Sbjct: 157 KKEVSSYIKKIGYNPAAVAFVPISGWHGDNMLEPSTKMPWFKGWLVERKEGKAEGKCLIE

Query: 183 ALDAILPPARPTDKPLRLXLXGRXQNRAVLVRCPXGXVKTGILKPGTXXXXXXXXXXXXS
           ALDAILPPARPTDKPLRL L    +   +    P G V+TGILKPGT
Sbjct: 217 ALDAILPPARPTDKPLRLPLQDVYKIGGI-GTVPVGRVETGILKPGTIVVFAPANITTEV

Query: 363 *VRGDAPRXPPGGCPGDNVGFNVKNVSVKELRRGYVXGDSXTTHPRVPADFPCSGXRTQP
                     PGDNVGFNVKNVSVKELRRGYV GDS    P+  ADF
Sbjct: 276 KSVEMHHEALQEAVPGDNVGFNVKNVSVKELRRGYVAGDSKNNPPKGAADFTAQVIVLNH

Query: 543 PGQXXNGXXPVLNCH 587
           PGQ  NG  PVL+CH
Sbjct: 336 PGQISNGYTPVLDCH 350
```

SK83

```
GTTATATGCTTGTCTCAAATTTTNAGCCATGCATGTCTCAGTGCAAGCCGNATTANTGCGATACCGCGAAT
GGCTCAATATATCAGTTTTGGTTCCTTAGATCTTACTCAGTTACTTGGATAACTGTGGTAATTCTAGAGCTA
ATACATGCAATCAGAACTCTGACCAGTGATGGGATGAGTGCTTTTATTAGATCAAAACCAATCGACGGATGG
CCATGTGTCTGAAGTCGTTAATTTTGATGAATCTGGATAACTTTTGCCNATCGCATGGCCAGTACCCGGCGA
CGCATCTTTCAAATGTCTGCCTTATCAACTTTCGATGGGAGGTTCTTGCGACTACCATGGGTGCACCGGGTA
CCGGGAATCAGGGTTCGATTCCGGAGANGGGAGCCTGAAAACCGGTTCCACATCCAAGGAAGNAGGAGGGGC
CCCAAATACCCCTTCCCGCACCGGGAGGGGGGGGACAAAAAAAACCATCCCGGACTNTTACCANGCCTCNNA
ATCGGAANGAGNCCCCTTTTAATNNTTTTAACCANGGACCCCACCTATCTNGG
```

Sequences producing significant alignments:	Score (bits)	E-Value
gi|18766078|gb|AF423786.1| Hyles lineata 18S ribosomal RNA ...	577	e-161
gi|18766076|gb|AF423784.1| Platyptilia sp. L11 18S ribosoma..	577	e-161
gi|11095921|gb|AF286273.1| Hemileuca sp. MFW-2000 18S ribos...	577	e-161
gi|1150401|emb|X89491.1|GMRRNA18S G.mellonella DNA for 18S ...	571	e-160
gi|18766077|gb|AF423785.1| Anthocharis sara 18S ribosomal R...	569	e-159
gi|11095947|gb|AF286299.1| Papilio troilus 18S ribosomal RN...	557	e-156
gi|11095946|gb|AF286298.1| Galleria mellonella 18S ribosoma...	557	e-156
gi|18766075|gb|AF423783.1| Prionoxystus robiniae 18S riboso...	545	e-152
gi|15021655|gb|AF394683.1| Danaus chrysippus orientis 18S r...	500	e-138
gi|15021654|gb|AF394682.1| Danaus chrysippus chrysippus iso...	500	e-138

```
Strand = Plus / Plus
Query: 2   ttatatgcttgtctcaaattttnagccatgcatgtctcagtgcaagccgnattantgcga
           ||||||||||||||||||  || ||||||||||||||||||||||||| ||||  ||||
Sbjct: 1   ttatatgcttgtctcaaagattaagccatgcatgtctcagtgcaagccgtattaaggcga

Query: 62  taccgcgaatggctcaatatatcagttttggttccttagatcttactcagttacttggat
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 61  taccgcgaatggctcaatatatcagttttggttccttagatcttactcagttacttggat

Query: 122 aactgtggtaattctagagctaatacatgcaatcagaactctgaccagtgatgggatgag
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 121 aactgtggtaattctagagctaatacatgcaatcagaactctgaccagtgatgggatgag

Query: 182 tgcttttattagatcaaaaccaatcgacggatggccatgtgtctgaagtcgttaattttg
           |||||||||||||||||||||||||||||| ||||  | ||| ||||||||||||||||
Sbjct: 181 tgcttttattagatcaaaaccaatcgacggagggcctcgcgtccgaagtcgttaattttg

Query: 242 atgaatctggataacttttgccnatcgcatgg-ccagtacccggcgacgcatctttcaaa
           |||||||||||||||||||||| ||||||||| |||||| ||||||||||||||||||||
Sbjct: 241 atgaatctggataacttttgccgatcgcatggtccagta-ccggcgacgcatctttcaaa

Query: 301 tgtctgccttatcaactttcgatgggaggttcttgcgactaccatgggtg-caccgggta
           |||||||||||||||||||||||| || ||| |||||||||||||| || ||| ||  |
Sbjct: 300 tgtctgccttatcaactttcgatggtagtttc-tgcgactaccatggttgtcacgggtaa

Query: 360 ccgggaatcagggttcgattccggagangggagcctga-aaaccggttccacatccaagg
           | |||||||||||||||||||||||| |||||||||| |||| | | ||||||||||||
Sbjct: 359 cggggaatcagggttcgattccggaga-gggagcctgagaaacggctaccacatccaagg

Query: 419 aag 421
           |||
Sbjct: 418 aag 420
```

SK86

GTTCCTCAAAGGAAAAGGTAAGAAGGCTGACATTGACCGTAGGGCTGAACAGATCCGTGACCAAATTCAGGA
AACAAGCTCTGAATATGAAAAGGAGAAGTTGCAGGAACGTCTTGCCCGACTTGCATCTGGTGTTGCTGTTTT
ACATGTCGGAGGATCCAGTGAAGTTGAGGTCAATGAGAAGAAGGATCGTGTCAATGATGCCCTGAATGCAAC
CCGTGCTGCTGTGGAGGAAGGTATTGTTCCCGGAGGTGGCTCTGCTCTCCTTAGATGTATCCCAGACCTTGC
AGGACTGAAGACTGCTAACAGTGACCAGGCCACAGGAGTAGAAATCGTCAGAAAGGCGCTCAGAATGCCCTG
CATGACTATCGCCCGTAATGCTGGTATTGATGGCTCAGTTGTTGTTGCTAAGGTTGAAGACTTAGGACCTGA
ATTTGGGTATGATGCGCTCAACAATGAATATGTCAACATGATTGAGAAGGGTATCATTGACCCCACCAAGGT
CGTAAGGACAGCACTGACTGCCCCAGTGGAGTAGCCGTCACTCCTCACCACCGCAGAAGCCGCATATGCGAA
ATCCACAAGAGAAGGACCCAANCCTATGGGTGGCATTGGAGGTATTGGCCGGATTGGTGGCATGGGAGGAAT
ATTA

Sequences producing significant alignments: Score (bits) E-Value

gi|2738077|gb|AAB94640.1| heat shock protein 60 [Culicoides...293 3e-78
gi|31231072|ref|XP_318461.1| ENSANGP00000014839 [Anopheles ...291 6e-78
gi|12644042|sp|O02649|CH60_DROME 60 kDa heat shock protein,...281 1e-74
gi|33636453|gb|AAQ23524.1| SD06594p [Drosophila melanogaster] 280 2e-74
gi|1653979|emb|CAA70287.1| 60kDa heat shock protein [Drosop...280 2e-74
gi|48103847|ref|XP_392899.1| similar to ENSANGP00000014839 ...280 2e-74
gi|6066606|emb|CAB58441.1| Hsp60 protein [Myzus persicae] 270 2e-71
gi|3757828|emb|CAA67720.1| heat shock protein 60 [Drosophil...267 2e-70
gi|116253|sp|P25420|CH63_HELVI 63 kDa chaperonin, mitochond...261 9e-69
gi|21064097|gb|AAM29278.1| AT16985p [Drosophila melanogaster] 260 1e-68

Frame = +2

Query: 5 LKGKGKKADIDRRAEQIRDQIQETSSEYEKEKLQERLARLASGVAVLHVGGSSEVEVNEK
LKGKG K IDRRAEQIRDQI+ET+S+YEKEKLQERLARL++GVA+L +GGSSEVEVNEK
Sbjct: 358 LKGKGTKEHIDRRAEQIRDQIKETTSQYEKEKLQERLARLSAGVALLRIGGSSEVEVNEK

Query: 185 KDRVNDALNATRAAVEEGIVPGGGSALLRCIPDLAGLKTANSDQATGVEIVRKALRMPCM
KDRV DALNATRAAVEEGIVPGGG+ALLRCIP L GLK N DQ TG+EIV +ALRMPCM
Sbjct: 418 KDRVTDALNATRAAVEEGIVPGGGTALLRCIPTLKGLKGENEDQKTGIEIVMRALRMPCM

Query: 365 TIARNAGIDGSVVVAKVEDLGPEFGYDALNNEYVNMIEKGIIDPTKVVRTALTAPVE*PS
TIA+NAG+DGSVVVAKVE+ E+GYDA+NNEYVNMIEKGIIDPTKVVRTALT S
Sbjct: 478 TIAKNAGVDGSVVVAKVEENQGEYGYDAMNNEYVNMIEKGIIDPTKVVRTALTDASGVAS

Query: 545 LLTTAEAAYAKSTRE 589
LLTTAEA + ++
Sbjct: 538 LLTTAEAVVTEMPKD 552

SK94

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CHAPTER 2

Role of juvenile hormone esterase and epoxide hydrolase in reproduction of the cotton bollworm, *Helicoverpa zea*

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Abstract

The role of juvenile hormone (JH) esterase (JHE) and epoxide hydrolase (EH) in reproduction of the cotton bollworm, *Helicoverpa zea*, was investigated. Peak emergence of male and female bollworm adults occurred early in the scotophase. Female adults were mixed with males in a 1:2 ratio, respectively, at the beginning of the first photophase after emergence. The highest oviposition rates for mated females were noted on d 2-4 at the level of 315±84 (±1 SEM), 368±80 and 338±43 eggs female^{-1} day^{-1}, respectively. Egg production for virgin females (reared in the absence of males) was 19.5±5.6, 17.7±7.8 and 56.6±10.7 eggs female^{-1} day^{-1}, respectively. The *in vitro* JH III esterase and JH III EH activity was measured in whole body homogenates of virgin and mated females from d1 to d8 post-emergence. Maximal JHE activity for virgin females occurred on d2 (1.09±0.14 (±1 SEM) nmol of JH III metabolized min^{-1} mg protein^{-1}), which was approximately twice that of mated females on the same day (0.49±0.04 min^{-1} mg protein^{-1}). The same results were observed for EH where the activity peaked on d2 at 0.053±0.003 as compared to 0.033±0.003 nmol of JH III metoblized min^{-1} mg protein^{-1}, respectively. By d4, both JHE and JH EH activities declined significantly in virgin and mated females and were the same between sexes through d7. The developmental changes and effects of mating on JH metabolic activity were similar when measured per insect. The highest levels of JHE and JH EH activity min^{-1} mg protein^{-1} in d2 virgin and mated females was found in ovaries followed by the carcass and then hemolymph; no EH activity was found in hemolymph. For ovary, the JHE and JH EH activity was highest in

virgin compared to mated females. The role of both enzymes in the regulation of reproduction is discussed.

1. Introduction

Juvenile hormone (JH) plays a role in almost all physiological, developmental, and reproductive processes in insects. JH titer is regulated by two main processes, synthesis and degradation (reviewed by Gilbert et al., 2000). One primary pathway for JH metabolism is hydrolysis of its methyl ester by highly specific juvenile hormone esterase (JHE) as indicated in many reports (Hammock, 1985; Roe and Venkatesh, 1990; de Kort and Granger, 1996). A second possible pathway for JH metabolism is the hydration of the 10,11-epoxide by epoxide hydrolase (EH).

JHE activity and its role in insect development were extensively studied in different life stages in insects. The role of JHE in embryogensis were first examined by Roe et al. (1987) and Share et al. (1988); larval development was studied by Hanzlik and Hammock (1988), Jesudason et al. (1990) and many others reviewed by Hammock (1985), Roe and Venkatesh (1990) and Gilbert et al. (2000); and reproduction was examined by Venkatesh et al. (1988), Cusson and Delisle (1996), Ramaswamy et al. (2000) and Cole et al. (2002). Using both the *in vivo* over expression of JHE with a Baculovirus expression system and JHE inhibition with slow, tight binding, transition state analogue inhibitors, it is clear that ester hydroylsis is important in the primary metabolism of JH, the regulation of JH titer and in the regulation of larval development and metamorphosis (reviewed by Gilbert et al., 2000). However in comparison to JHE, much less is known

about the regulation and function of EHs in insects. Some studies indicated that EH plays a role in xenobiotic metabolism as reported by Slade et al. (1975), Brooks (1977) and Mullin (1988). Recently, Taniai et al. (2003) found that a clofibrate-inducible microsomal EH from *Drosophila melanogaster* metabolized cis-stilbine oxide but not JH III. 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) suggested that EHs might also be important in the regulation of JH titer in insects including the Lepidoptera.

Increases in adult juvenile hormone titer appears to regulate vitellogensis in insects. This increase in JH titer can result from an increase in the rate of JH biosynthesis by the corpora allata (CA), decreases in JH esterase activity, and the transfer of JH from the male to the female during copulation. There have been a number of reports of the activation of the female CA after mating to produce JH. For example, Herman and Parker (1977) found that the CA was inactive in virgin female monarch butterflies, *Danaus plexippus*. When the insect was allowed to mate 10 days after emergence, the CA produced JH and eggs were developed. Stay and Tobe (1977) reported that mating was necessary for activation of the CA in the female cockroach, *Diploptera punctata*, and Park et al. (1998) reported increased JH production by the CA after mating in female adults of the tobacco budworm, *H. virescens*. The initiation of JH synthesis by the CA may originate from factors synthesized in the male accessory glands and transferred to the female during copulation in *Drosophila melnogaster* (Moshitzky et al., 1996) or in the case of the tobacco hornworm, *Manduca sexta*, by allatropin produced by the brain in

response to mating induced neural signals from the bursa copulatrix (Sasaki and Riddiford, 1984). Transfer of JH from the male to the female during copulation was reported first in *Hyalophora cecropia* by Shirk et al. (1980). Bhaskaran et al. (1988) showed that the accessory glands of some male Lepidoptera contained JH acid methyltransferase that can convert JH acid to JH, and this JH is transferred to the female during mating. Also, Park et al. (1998) reported that male *H. virescens* transferred JH to the female during mating.

Venkatesh et al. (1988) were the first to find a reduction in JHE activity in the hemolymph of adult female cabbage loopers, *Trichoplusia ni*, after mating. The activity remained elevated in virgin females, presumably in support of a low JH titer and reduced egg production until after mating. Ramaswamy et al. (2000) also reported the same for female adults of the tobacco budworm, *H. virescens*. In the tobacco budworm adult it appears that increases in JH biosynthesis by the female, the transfer of JH from the male to the female and decreases in JHE activity are responsible for the initiation of vitellogenesis. However, in the torticid moth, *Cydia pomonella*, Cole et al. (2002) found no difference in JHE activity between virgin and mated females and concluded that JHE was not playing a role in reproduction. Cusson and Delisle (1996) reported that although JH levels in mated was higher than that of virgin females of the torticids, *Choristoneura fumiferana* and *Ch. rosaceana*, JHE activity was also higher in mated females as compared to virgins. Cusson et al. (1999) and Delisle and Cusson (1999) showed that males sex accessory glands could not synthesize JH, JH was not transferred to females

during copulation and there were no differences in CA activity between virgin and mated females.

No studies have been conducted to examine the possible effects of mating on the regulation of JH EH activity in the Lepidoptera and the possible role of changes in JH EH activity in the regulation of reproduction. In addition the role of EHs in general in JH metabolism especially in the Lepidoptera has been in question because of a lack of an *in vivo* JH EH inhibitors; in addition no *in vivo* EH expression studies have been conducted (Gilbert et al. 2000). The current study examines the correlation of mating in adult females of the cotton bollworm, *Helicoverpa zea*, with JH EH activities in the whole insect and specific tissues at different developmental ages and in correlation with changes in the JH esterase activity in order to better understand the role of epoxide hydrolases in the regulation of JH and its possible role in reproduction.

2. Materials and methods

2.1. Insects, biology and sample collection

Cotton bollworm pupae were obtained from the North Carolina State University insectary. This strain was originally collected from cotton plants in Plymouth, NC in August and September of 1996 and reared continuously on artificial diet. Male and female pupae were maintained separately at 27±1 ºC with a 14:10 h light:dark cycle (lights on at 8 am Eastern Standard Time) and at 50-60% relative humidity until emergence. A 5% contamination of the cotton bollworm colony with tobacco budworm, *Heliothis virescens*, adults was found when voucher specimens were examined after the

completion of the research; this contamination occurred only for the studies on JH metabolism in hemolymph, ovaries and carcass (described later). To determine the peak time of emergence, the presence of new adults was observed every two hours during the photoperiod; during the dark cycle, the insects were exposed to low levels of light for about 15 sec to observe the adult moths. Peak adult emergence occurred during the scotophase, and newly eclosed moths at this time were designated as day 0 in adult age. Day 0 male and female adults were transferred to a 1-gallon plastic container in the ratio 2:1, respectively; the assumption was made that these females were eventually 100% mated. This was further validated by the observation of high egg production from these females. Sides of the container were lined with paper towels (Fort James Corp, Deerfield, IL) and the open top covered with 1 layer of cheese cloth. Adults were fed on a honey:distilled water (1:1) mixture and maintained at the standard rearing conditions defined earlier. Approximately one fourth of the hind wing on both sides of female moths was removed by cutting with scissors in order to distinguish these insects from the males. Unmated females (referred to as virgin females) in our study were reared in the absence of males. After emergence, mated and virgin females were collected every 24h (starting with day 1) for 8 d and stored at -80 ºC until needed for analysis. Storage at -80 ºC had no effect on the JHE and JH EH activity or protein determination.

To measure the oviposition rate for mated and virgin moths, cohorts of 1 female and 2 males or 1 female alone were transferred to separate 250 ml plastic containers and maintained under standard rearing conditions. The moths at the time of transfer were 0 days old. The opened end of the container was covered with cheese cloth and served as

the oviposition substrate. The cloth was removed every 24 h through 10 d after emergence, and the number of oviposited eggs counted.

Hemolymph from mated and virgin females 2 d after emergence was collected through a small incision made on the dorsal surface of the abdomen. Hemolymph was centrifuged for 5 min at 1000 *g*, and the plasma stored at -80 ºC until needed for analysis. Ovaries were dissected from mated and virgin females of the same age with their antennae, wings and legs removed. After removal of the ovaries, the remaining carcass was flushed with sodium phosphate buffer (0.1 M, pH 7.4, 0.01% phenylthiourea) to remove any residual hemolymph. Ovaries were also washed with buffer. Samples of ovary and carcass were stored at -80 ºC until needed for further analysis.

2.2. Preparation of homogenates

Before homogenization of whole moths, their wings, antennae and legs were removed and the remaining moth weighed. The moth (without their legs, antennae and wings) or carcasses (described earlier) were homogenized for 1 min in ice-cold sodium phosphate buffer (0.1 M, pH 7.4, 0.01% phenylthiourea) at the rate of 1 g body weight/4 ml buffer using a Polytron PT10/35 homogenizer (Brinkmann Instruments, Westbury, NY; speed 6). Ovaries were homogenized with a Kontes Duall (0.3 ml working capacity) glass-on-glass tissue grinder (Kontes Glass Co., Vineland, NJ) using the same proportion of buffer to tissue as that used for whole body and carcass. Homogenates were centrifuged at 10,000 *g* for 10 min at 4 ºC, and the supernatants were centrifuged a

second time under the same conditions. The final supernatant was stored at -80 ºC until needed for analysis.

2.3. Determination of JH metabolites and JHE inhibition by 3-octylthio-1,1,1 trifluoropropan-2-one (OTFP)

JH metabolism in whole body homogenates in the presence and absence of OTFP was determined by thin layer chromatography (TLC) as described by Share and Roe (1988). Body homogenates from d 3 virgin females were used for this analysis. OTFP, a potent and highly specific slow, tight binding JHE inhibitor, was synthesized according to Linderman et al. (1987). For all JH metabolism studies, the substrate was a mixture of [^{3}H]-racemic JH III (12 Ci/mmol, tritiated at C10; PerkinElmer Life Sciences, Boston, MA) and unlabeled racemic JH III (Sigma, Milwaukee, WI). The final concentration of JH III substrate was $5x10^{-4}$ M in absolute ethanol having ~ 8000 cpm/µl. One ml of body homogenate clarified by centrifugation was preincubated for 10 min at 30 ºC in 13 x 100 mm test tube with either 10 µl of absolute ethanol (for total JH metabolism) or 10^{-2} M OTFP in ethanol (final OTFP concentration 10^{-4} M). Assays with OTFP measure total JH metabolism except that of JHE. JH III substrate (10µl, previously described) was then added to the homogenates (final JH substrate concentration of $5x10^{-6}$) and the mixture incubated for 30 min at 30 ºC. After this second incubation, sodium chloride was added to the reaction until saturation, followed by the addition of 1 ml of ethyl acetate. The reaction mixture was then vortexed vigorously to mix the two phases and centrifuged for 5 min at 1000 *g* at room temperature. The top ethyl acetate layer was transferred to a screw-cap, teflon-sealed vial. Extraction with ethyl acetate was repeated two more times

producing a total of 3 ml of an ethyl acetate extract (final volume in the teflon-sealed vial). The volume was reduced to ~100 µl at 35 ºC in a water bath.

For TLC, Polygram SILG, (0.25 mm thick and 5 x 20 cm dimensions) silica gel plastic plates (Brinkmann) were activated at 100 ºC for 30 min. Each 100 µl ethyl acetate extract was then spotted in separate lanes 1.5 cm from the bottom of the TLC plate. The TLC plate was then developed in ethyl acetate:hexane:glacial acetic acid (33:33:1) followed by air drying. The plate was then cut into 0.5 cm fractions starting at the origin and ending at the solvent front. Each fraction was assayed separately by liquid scintillation counting. JH III metabolites were identified by comparing the R_f values for each metabolite with the standard values reported in Share and Roe (1988).

2.4. JHE and JH EH metabolic activity

Plasma and homogenates were assayed for JH III esterase and JH III epoxide hydrolase activity as described by Share and Roe (1988). Diluted samples (100µl) were incubated first with 1µl ethanol (for total metabolism) or 1µl of 0.01 M OTFP (for EH metabolism) in 10 x 75 mm test tubes at 30 ºC for 10 min. JH III substrate (1µl, previously described) was then added to each reaction, and tubes were incubated at 30 ºC for 15 min. The reaction was quenched with methanol (300 µl) and isooctane (250 µl). Tubes were vigorously vortexed and then centrifuged at 1000 *g* at room temperature for 5 min to separate the organic and aqueous phases. Aliquots of 100µl from the top (organic) and bottom (aqueous) phases were analyzed by liquid scintillation. Enzyme concentrations and incubation times were chosen that produced a linear increase in

percent metabolism versus time and which were directly proportional to the protein concentration. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, 1977) using bovine serum albumin (Fraction V; Fisher Scientific, Pittsburgh, PA) as a standard.

3. Results

3.1. Adult emergence

Adult emergence was observed every 2h during the dark and light periods. Most of the adults, approximately 70% of the females and 80% of the males, emerged in the first 4h of the scotophase (Fig 1 A and B, respectively). Only minimal emergence (12% and 8%, respectively) occurred during the photophase. Those insects that emerged during each dark cycle were considered to be Day 0 moths at the beginning of the photophase for the studies that follow. Adults emerging during the photophase were not used in our studies.

3.2. Oviposition

The daily rate of egg laying for virgin versus mated females of the cotton bollworm from d 0 through d 10 is shown in Fig. 2. On d1, the average number of eggs oviposited was 16$\pm$4 ($\pm$1 SEM) and 26$\pm$10 per female per day for virgin versus mated moths, respectively, with no statistically significant differences between treatments (t-test, $P < 0.05$). For mated females by d 2, the number of eggs per female per day increased significantly to 315$\pm$84. Oviposition peaked on d 3 to 368$\pm$80 and then began to decrease

to 338±43 by d 4. By d5, egg production was 151±25 and the decrease continued to 34±6 by d10. For virgin females, the oviposition rate was unchanged from d 1 through d 3 and was minimal during the time period of peak egg production in mated females. There was a modest increase in the oviposition rate in the virgin females after d 3 which peaked on d 8 at 126±23 eggs/female/day and then decreased thereafter. The oviposition rate was the same between mated and virgin females from d 7 through d 10. The total number of eggs produced per female from d 1 through d 10 was 526±50 and 1879±110 for virgin versus mated moths, respectively.

3.3. JH metabolites and JHE inhibition by OTFP

The *in vitro* metabolism of JH III in clarified whole body homogenates of virgin d 3 *H. zea* was determined by thin layer chromatography (TLC). In the absence of OTFP, four radiolabeled compounds were resolved above the origin. These were identified by their R_f values as JH III (R_f, 0.76), JH III acid (0.56), JH III diol (0.25), and JH III diol acid (0.13)(Fig. 3). JH III incubations in the presence of 10^{-4} M OTFP produced only two compounds above the origin, JH III (R_f, 0.77) and JH III diol (0.24). In both treatments, no radioactivity was found at the origin.

3.4. Effect of mating on JHE and EH activity in the whole body

JH III esterase and JH III EH activity were measured daily from d 1 to d 8 (Figs. 4 and 5). On d 1, there was no difference between virgin and mated females in both enzyme activities expressed as nmol JH III metabolized/min/mg protein or as nmol JH III

metabolized/min/insect (t-test, $P < 0.05$). On d 2 in virgin females, the activity of both enzymes was at its highest level than at any other time after emergence. For virgin females the JHE activity was 1.09±0.14 (±1 SEM) nmol JH III/min/mg protein or 11.22±0.56 nmol/min/insect, for EH the activity level was 0.053±0.003 and 1.006±0.106, respectively. While the JHE and JH EH activity increased from d 1 to d 2 in virgin moths, both decreased in mated females. The d 2 JHE activity in mated females was 0.494±0.045 nmol JH III/min/mg protein or 5.574±1.402 nmol/min/insect, for EH the activity level was 0.033±0.003 or 0.476±0.05, respectively. From d 2 through d 4, there was a decline in JHE and JH EH activity in both virgin and mated moths so that by day 4 there were no differences in the *in vitro* JH metabolic activity as a result of mating. The JH esterase activity, as well as the JH EH activity, was mostly the same for mated and virgin adults from d 4 through d 8. At each time point examined after emergence for both virgin and mated females, the *in vitro* JH esterase activity was higher than the JH EH activity.

3.5. Effect of mating on JHE and JH EH activity in ovary, plasma and carcass

In vitro JH III metabolism in ovary, plasma and carcass of d 2 virgin and mated females of the cotton bollworm was determined. Both JHE (Fig. 6) and EH (Fig. 7) activities were higher in the ovary of virgin as compared to mated females. JH esterase activity in ovaries was 2.2±0.07 and 1.7±0.07, in carcass was 0.39±0.04 and 0.24±0.05 and in plasma was 0.15±0.002 and 0.11±0.01 nmol JH III/min/mg protein for virgin and mated females, respectively. When JH esterase activity was measured per unit volume, it

was much higher in virgin plasma as compared to the mated one where it was 18.55±1.39 and 7.36±0.64 nmol JH III/min/ml for virgin and mated females, respectively. The JH III EH activity in ovaries was 0.157±0.006 and 0.117±0.005 and in carcass was 0.0197±0.001 and 0.013±0.001 nmol JH III/min/mg protein for virgin and mated females, respectively. No EH activity was found in plasma. JH metabolism (both JHE and EH) was the highest in the ovaries and lowest in plasma.

4. Discussion

4.1. In vitro routes of JH metabolism in female adults of H. zea

JH III was incubated with d 3 whole body homogenates (wings, antennae and legs removed) of virgin female adults of *H.* zea. In the absence of the JH esterase inhibitor, OTFP, the only JH III metabolites that were resolved on TLC with almost identical Rf values as those previously reported in the literature (Share and Roe, 1988) were JH III acid, JH III diol, and JH III diol acid (Fig. 3). These products are the result of enzymatic JH esterase and JH epoxide hyhdrolase activity and are typical of most insect studies (Roe and Venkatesh, 1990; Gilbert et al., 2000). No activity was found at the origin of the TLC plate, which would be expected for JH III conjugates like that with glutathione. The production of diepoxides and tetrahydrofuran diols would not be expected in these assays because of the absence of a P450 regeneration system.

OTFP is a highly specific inhibitor of insect JH esterases. Although the inhibitor has not been previously tested against JH esterases from *H. zea*, OTFP is known to be an effective *in vitro* and *in vivo* inhibitor of this enzyme in other noctuids (Hammock et al.,

1984; Share and Roe, 1988; Gilbert et al., 2000). It was clear that OTFP at a final concentration of 0.1 μM, inhibited 100% of the JH esterase activity in homogenates from d 3 virgin adult females of the cotton bollworm (Fig. 3). This developmental time and condition was chosen because JH metabolism was at peak levels and would be the most difficult to inhibit. The successful demonstration of complete JH esterase inhibition was required to validate the Share and Roe (1988) partition assay used in our developmental studies. OTFP at 0.1 μM has no affect on JH epoxide hydrolase activity in insect homogenates previously studied (Share and Roe, 1988).

4.2. Role of JH esterase and JH epoxide hydrolase in H. zea reproduction

In this study we assessed the activity of JH III esterase and JH III epoxide hydrolase in virgin and mated *H. zea* females 1-8 d post emergence. Adult emergence in our studies was predominant during the scotophase (Fig. 1). Male and female moths that emerged in separate containers were considered d 0 in age at the time of peak emergence. The developmental age of the adults were measured in 24 h intervals from d 0. Mated females were produced by placing d 0 females (at lights-on) with d 0 males (at lights-on) in the ratio of 1:2, respectively; virgin females were reared in the absence of males. On day 1, there was no difference in egg production between mated and virgin moths but by day 2, oviposition on the average was greater than 300 eggs/female/day for mated versus 17 eggs/female/day for virgins, with minimal variation around the means (Fig. 2). The assumption was made that the majority of the females reared in the presence of male moths were mated, which appears to be validated by our ovipositional results (Fig. 2).

The eggs produced by mated female moths hatch normally into viable larvae; eggs produced by virgin moths did not hatch.

On d 1 after adult emergence, there was no difference in JHE activity between virgin and mated females when activity was expressed per mg protein and per insect (Fig. 4). This was expected since no mating activity was observed during this period, the ovipositional rate was minimal, and there were no differences in the number of eggs oviposited per female per day between mated and virgin females. On d 2, the JHE activity in virgin females was higher than that of mated females (Fig. 4) and was positively correlated with a low and high ovipositional rate, respectively (Fig. 2). Venkatesh et al. (1988) in studies with cabbage loopers and Ramaswamy et al. (2000) in studies with the tobacco budworm, found elevated JH esterase activity per unit volume in hemolymph of virgin as compared to mated females shortly after mating. The current study with the cotton bollworm, indicates that the same elevation in JHE activity occurs in whole body homogenates and in plasma (per unit volume). However, Cole et al. (2002) showed that there were no differences in the JHE activity levels per unit volume of hemolymph in virgin versus mated females of *C. pomonella*, and Cusson and Delisle (1996) showed that JHE activity in the hemolymph of *Ch. fumiferana* and *Ch. rosaceana* mated females was higher than that in virgin females.

In *T. ni* virgin females, hemolymph JHE activity remains high until d 10 while in mated females there is a dramatic drop in the JHE activity after mating (Venketesh et al., 1988). This was different from the results for *H.* zea, where whole body JHE activity per mg protein and per insect peaked on d 2, declined from d 2-4 and remained unchanged

from d 4-7 in virgin females (Fig. 4). For mated bollworms, there was a decline in activity during d 1-4 or 5 on a per mg protein or per insect basis, respectively, and then activity remained constant through d 8. Cusson and Delisle (1996) noted that JHE activity per unit hemolymph in virgin and mated *Ch. fumiferana* and *Ch. rosaceana* females declined between D1 and D5 after emergence.

Changes in JH epoxide hydrolase activity relative to lepidopteran reproduction have not been studied previously in Lepidoptera. Epoxide hydrolase activity per mg protein and per insect in whole body homogenates of the cotton bollworm followed similar patters of change to that of JHE (Fig. 5). EH activity on d 1 was the same in both virgin and mated females. This activity increased on d 2 in virgins and then declined thereafter, while there was a general decline in whole body JH EH activity after d 1 in mated females. These results represent the first report that JH III EH activity is affected by mating in female moths.

It appears that JH is important in the regulation of egg development and oviposition in adult insects including members of the family Noctuidae. Satyanarayana et al. (1991) could not find a measurable difference in the JH synthetic activity in isolated CA from virgin versus mated *H. zea* females, but concluded that the CA might be stimulated by the brain to produce and /or release JH after mating *in vivo*. They also showed that JH regulates egg maturation and oviposition in *H. zea.* Satyanarayana et al. (1992) through decapitation and methoprene (a JH analogue) injection, showed that vitellogenesis in *H. zea* is under the regulation of JH. Zeng et al. (1997) reported the same observation for *H. virescens*. Stimulation of JH biosynthesis by the CA after mating was reported in female

adults of *H. virescens* by Park et al. (1998). Moshitzky et al. (1996) found that sex peptides produced in the male accessory glands of *D. melanogaster* were able to increase JH biosynthesis in the isolated CA from virgin females, and this mechanism might be responsible for the initiation of egg development in the adult female. Fan et al. (1999, 2000) also showed that these male sex peptides could activate the CA in both *D. melanogaster* and in the lepidopteran, *H. armigera*. Jin and Gong (2001) isolated a protein from *H. armigera* male accessory glands. When this protein was injected into virgin female adults, oogenesis and oviposition was induced. There is also evidence that JH can be transferred from the male to the female during mating as reported by Shirk et al. (1980) in *Hyalophora cecropia* and Park et al. (1998) for *H. virescens*. In addition, Bhaskaran et al. (1988) found that the male reproductive system of *H. zea* contained JH acid methyltransferase, which converted JH acid produced by the CA into JH. The female reproductive system did not have this capability.

It appears as summarized by Ramaswamy et al. (1997), three mechanisms may be responsible for higher JH levels in mated females as compared to virgin females of *H. virescens* , i.e., increases in JH biosynthesis in the CA of mated females, the transfer of JH from the male to the female during copulation, and reduced hemolymph JH esterase activity. Reductions in the hemolymph JHE activity after mating was also reported in *T. ni* by Venkatesk et al. (1988) and in *H. virescens* by Ramaswamy et al. (2000). Although there are some differences in the pattern of changes between hemolymph JH esterase activity in *H. virescens* and *T. ni* as compared to that in whole body homogenates of female adults of *H. zea*, it appears that changes in the level of whole body JH esterase

activity might be important in reproduction of the cotton bollworm. Furthermore, it appears that changes in JH metabolism as the result of mating is not limited to that of JHE. JH III EH activity was also higher in whole body homogenates of virgin versus mated adult moths.

To examine this question further, JHE and JH EH activity was measured in the ovaries, hemolymph and remaining carcass of d 2 virgin versus mated cotton bollworm adults (Figs. 6 and 7). Day 2 moths were used because this was the developmental time during which differences in JH metabolism in whole body homogenates was the greatest between virgin and mated bollworms. Apparently the elevated JH III esterase and JH III epoxide hydrolase activity found in whole body homogenates was the result of elevated JHE activity and JH EH activity in the ovary of virgin over that of mated females. It appears that not only does mating result in a reduction in JH esterase activity in hemolymph like previously reported by Venkatesh et al. (1988) for *T. ni* and by Ramaswamy et al. (2000) for *H. virescens*, but the JH metabolic activity in tissues other than hemolymph are also affected.

It is interesting to note that the JHE and EH activity was higher in ovaries than in carcass for both virgin and mated *H. zea* (Figs. 6 and 7). This was also true for JHE in the ovaries as compared to hemolymph. Several reports have indicated that high levels of JH or JH mimics are lethal to young embryos (Riddiford and Williams, 1967; Riddiford. 1970; Gilbic and Sehnal, 1973; Cole et al., 2002). Roe et al. (1987) and Share et al. (1988) found high levels of JHE in preovipositional and newly laid eggs of *Acheta domestica* and *M. sexta*, respectively. They hypothesized that high levels of JHE were

necessary early in embryogenesis to clear any JH that might be transferred from the mother to the egg (Gilbert and Schneiderman, 1961; Temin et al., 1986). It is possible that the high levels of JHE and JH EH activity in the ovaries as compared to the other tissues examined (Figs. 6 and 7) are there to metabolize maternal JHs and limit the amount of JH entering the egg. However, it is interesting to note that although the level of JHE and JH EH activity in the ovary was significantly greater than the other tissues examined on a per mg protein basis, the levels were lower in the mated versus virgin females. The opposite might be expected for the ovaries, if the function of the JH metabolic activity at this site is to protect the eggs from maternal JH. This argues that the ovary activity is important also in the whole body reduction in JH metabolism as a result of mating. Changes in the whole body and specific tissue JH EH activity in response to mating as well as the positive correlation of changes in EH activity with that for JHE activity, suggest that epoxide hydrolases may have a role in the regulation of JH titer and female reproduction.

4.3. Role of JH esterase versus JH EH in JH metabolism and reproduction

The majority of information available about JH metabolism was derived from studies of hemolymph JH esterase. 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 regulated in part by JH esterase (Roe and Venkatesh, 1990; Jones et al., 1990; Roe et al., 1997; Gilbert et al., 2000). 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 especially in the Lepidoptera (Hammock, 1985). Despite this earlier conclusion, 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 JHE activities were correlated (Yu and Terriere, 1978). More recently, similar correlations were found in at least some tissues of the tobacco hornworm larva, *Manduca sexta* (Jesudason et al., 1992) and *T. ni* (Wing et al., 1981; Kallapur et al., 1996). Furthermore, Kallapur et al. (1966) discovered that peak levels of JH III EH activity were nearly equal to peak levels of JH esterase activity when measured in vitro in *T. ni* fat body, midgut and integument during the fifth stadium. Hanzlik and Hammock (1988) showed that whole body JH EH and JHE activity were roughly equivalent in third and fourth stadium larvae of the cabbage looper. Perhaps the most convincing evidence for a primary role for EH in the *in vivo* metabolism of JH was presented by Halarnkar et al. (1993). They discovered that a phosphate conjugate of JH diol, not JH acid-diol, was the principal end product when JH I was injected into larval *M. sexta*. Unlike JH esterase, however, the determination of a direct role for JH EH in lepiodpteran development via JH degradation has remained elusive, mainly due to the lack of selective *in vivo* EH inhibitors (de Kort and Granger, 1996).

Changes in the JH III esterase and JH III epoxide hydrolase activity during adult development and in response to mating was similar in the current study (Figs. 4 and 5), suggesting that both are important in the regulation of JH titer and reproduction in *H. zea*.

However, the JHE activity was between 14 to 20 times greater than that for the EH activity (per mg protein and per insect) indicating that JHE might be more important than EH in metabolism of JH in adult *H. zea*. Although the current study provides the first evidence that EHs might play a functional role in the regulation of adult reproduction in the Lepidoptera like that of JHE, it is not yet clear whether epoxide hydrolase is involved in the metabolism of JH or JH acid. In addition, it is not clear as to the significance of epoxide hydration as compared to JH esterase relative to the regulation of egg development and oviposition.

In summary, the current study found that similar changes in the JH esterase and JH epoxide hydrolase activity occurred during adult development and in response to mating in the cotton bollworm. It appears that increased ester hydrolysis and epoxide hydration contribute to low levels of JH and low levels of oviposition in virgin adults; the reverse occurs after mating. The increased levels in virgin females are found in hemolymph (activity/ml) and ovaries (activity/mg protein) for JHE and in ovaries for JH EH. The JHE activity was higher than the JH EH activity at all developmental times examined, in both virgin and mated moths, and in all tissues examined suggesting a greater role for JHE in the regulation of JH titer.

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FIGURES

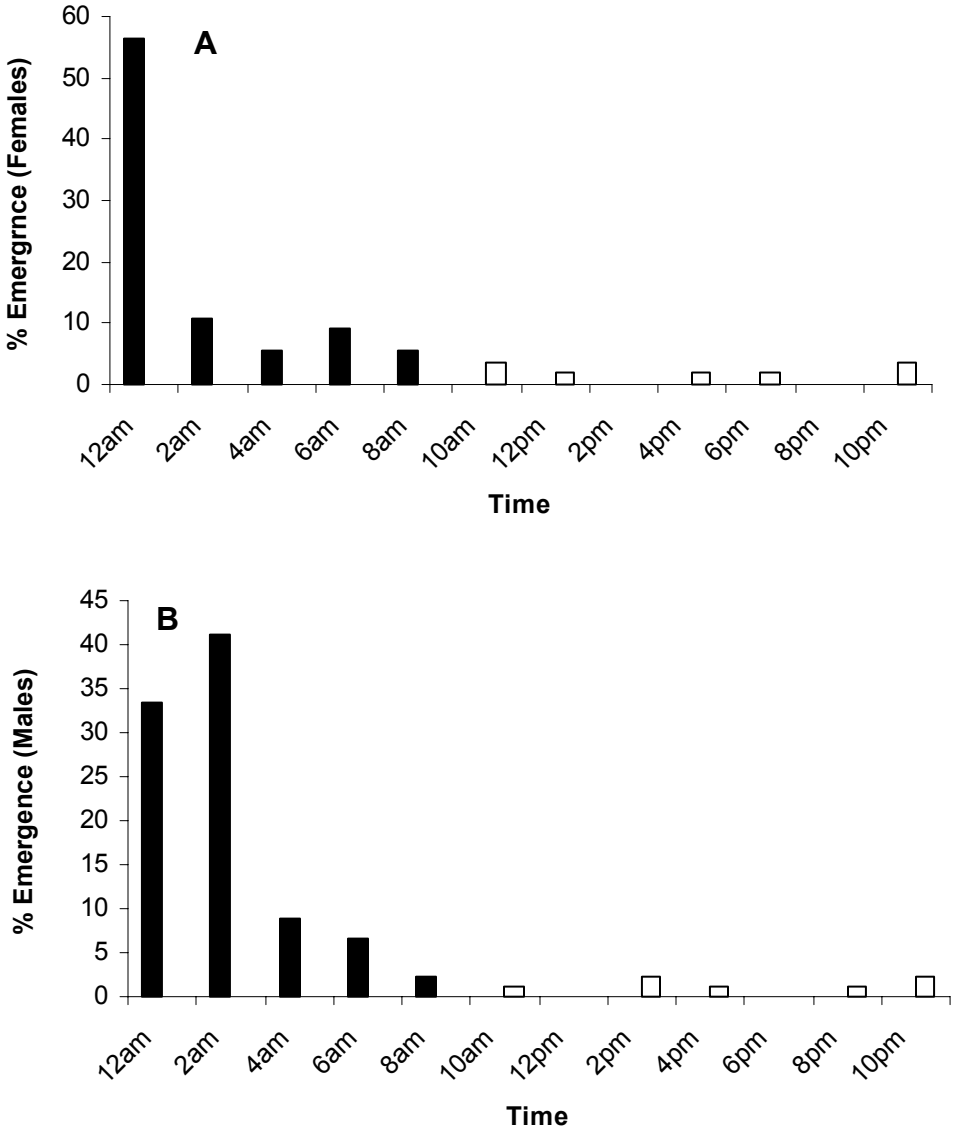

Fig. 2.1. Percentage of adult emergence in relation to the light-dark cycle (LD 14:10) for (A) females (n = 145) and (B) males (n = 163) of the cotton bollworm, *Helicoverpa zea*. Solid bars represent the scotophase. Adults emergence was recorded over 2 h intervals.

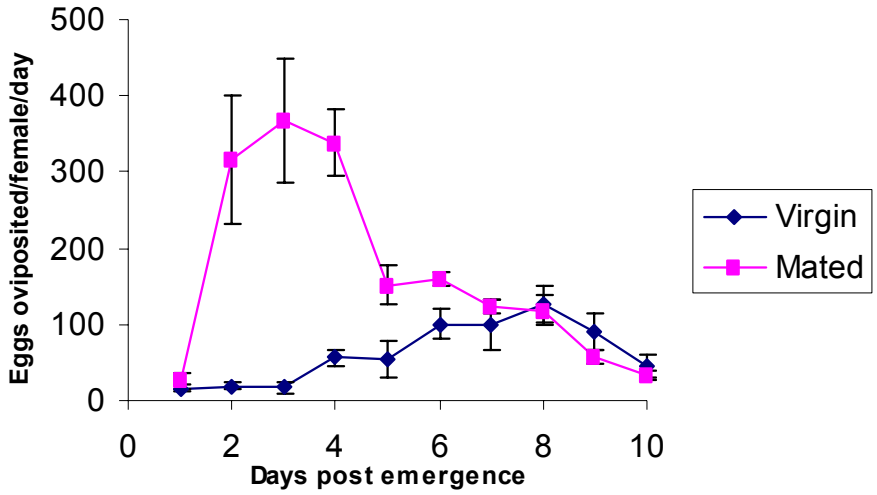

Fig. 2.2. Number of eggs oviposited daily by virgin and mated females of *H. zea*. Eggs counted on d 1 were those oviposited from the time of emergence to d 1, those on d 2 are those oviposited between d 1 and d 2. The data plotted is the mean $\pm$1 SEM for 5-6 females for each day post emergence.

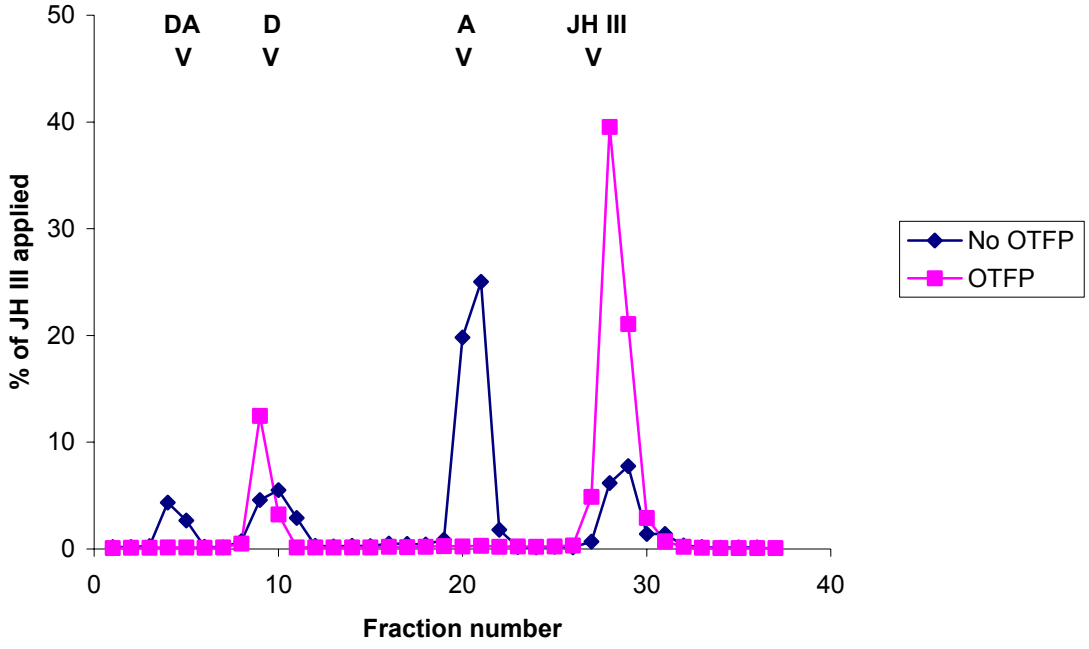

Fig. 2.3. JH III metabolism with and without the JH esterase inhibitor OTFP (10^{-4} M, final concentration) in clarified body homogenates from d 3 virgin females of *H. zea*. Total *in vitro* JH III metabolism is measured in the absence of OTFP. A, JH III acid; D, JH III diol; DA, JH III diol, acid; JH III, juvenile hormone III; OTFP, 3-octylthio-1,1,1 trifluoropropan-2-one.

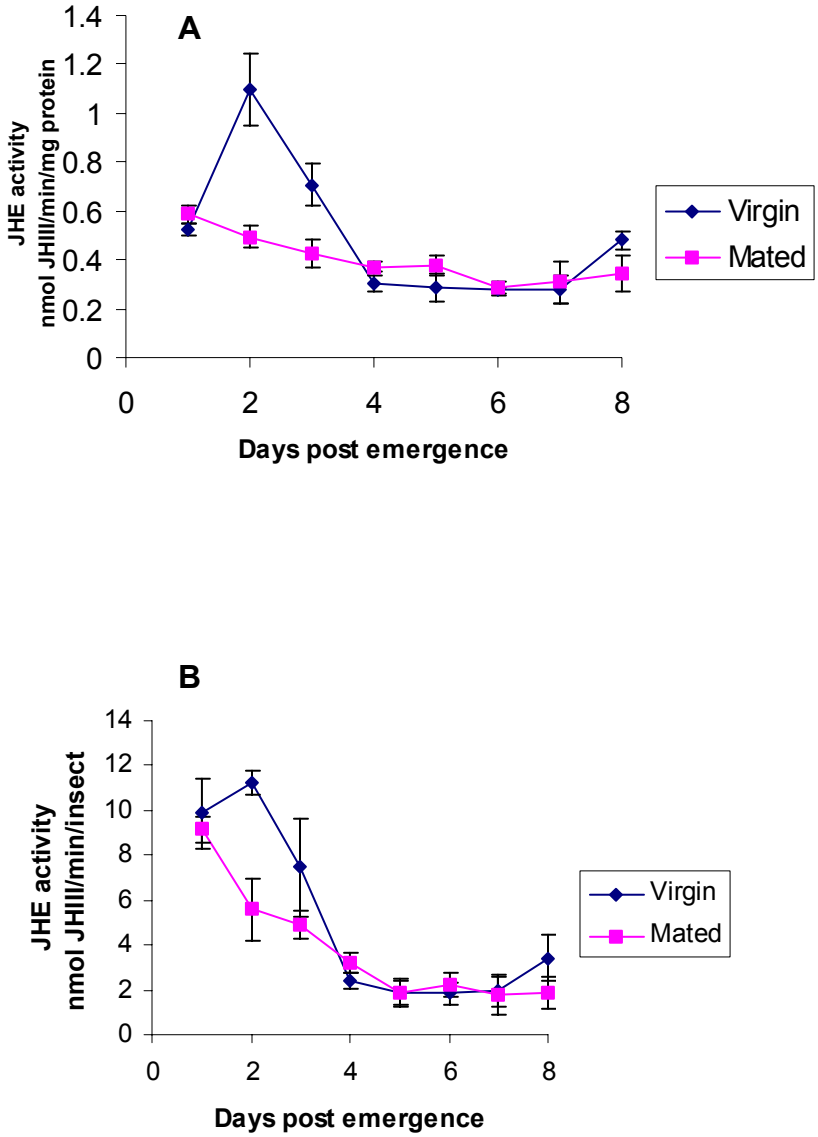

Fig. 2.4. JH III esterase (JHE) activity in whole body homogenates of virgin and mated females of *H. zea* from d 1 through d 8 after emergence. A, JHE activity/min/mg protein; B, JHE activity/min/insect. Each data point plotted is the mean of five replicates with three insects per replicate ±1 SEM.

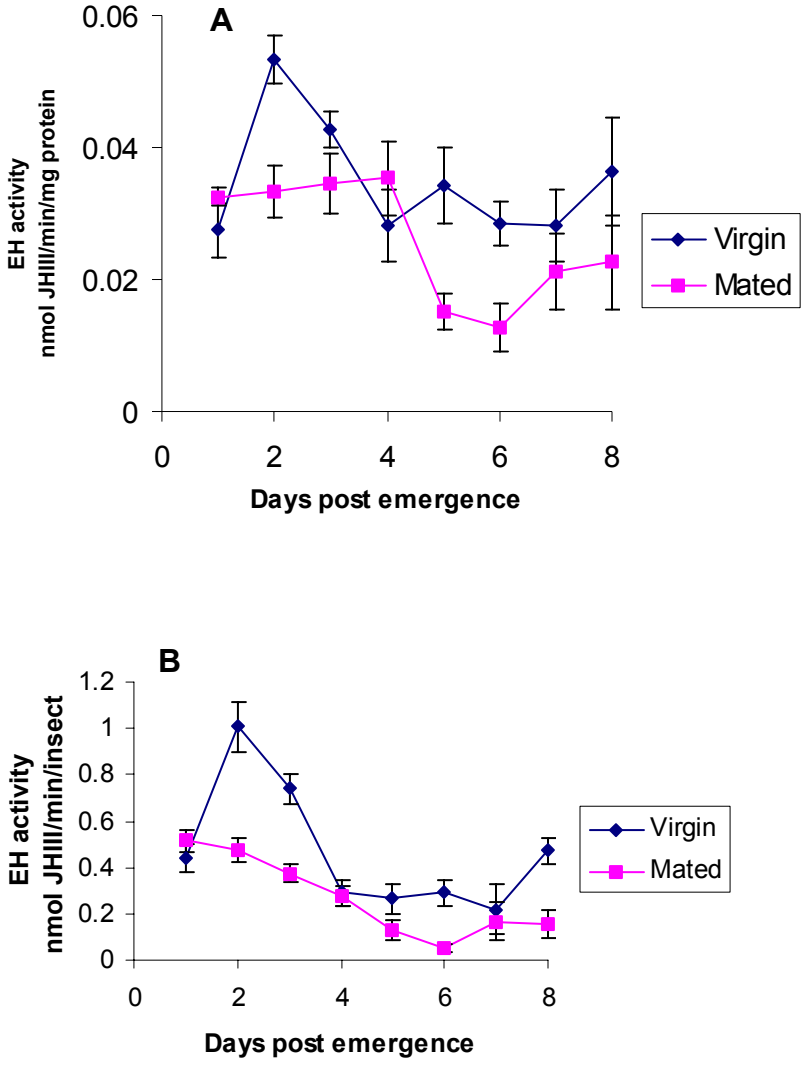

Fig. 2.5. JH III epoxide hydrolase (EH) activity in whole body homogenates of virgin and mated females of *H. zea* from d 1 through d 8 after emergence. A, EH activity/min/mg protein; B, EH activity/min/insect. Each data point plotted is the mean of five replicates with three insects per replicate $\pm$1 SEM.

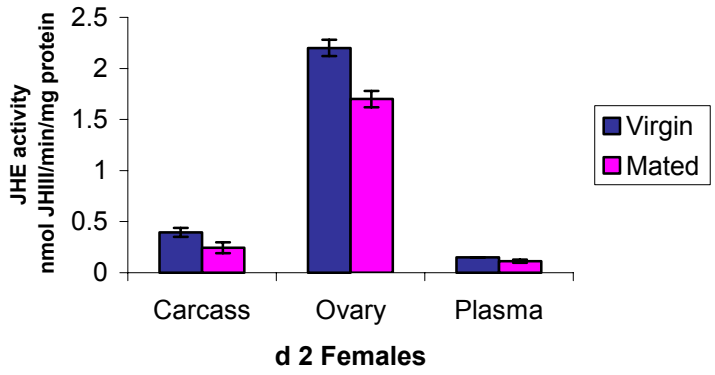

Fig. 2.6. JH III esterase (JHE) activity in ovary, hemolymph and carcass of virgin and mated d 2 females of *H. zea*. Each data point plotted is the mean of five replicates with three insects per replicate $\pm$1 SEM.

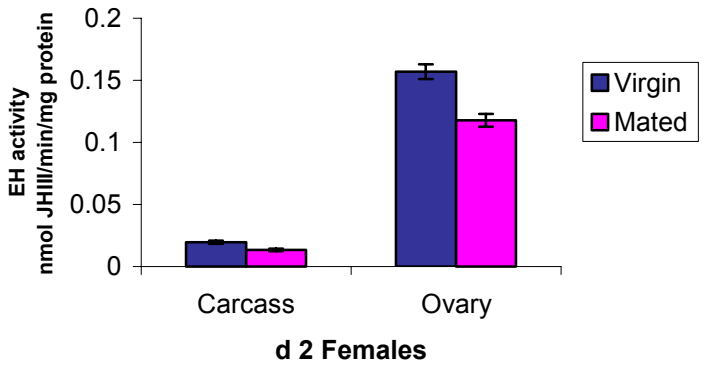

Fig. 2.7. JH III epoxide hydrolase (EH) activity in hemolymph and carcass of virgin and mated d 2 females of *H. zea*. Each data point plotted is the mean of five replicates with three insects per replicate $\pm$1 SEM.

CHAPTER 3

The Use of the InsectSelect Glow system for the expression of insect microsomal epoxide hydrolase in insect cell lines

Abstract

In insects, epoxide hydrolases (EHs) are believed to play a role in xenobiotic transformation and juvenile hormone metabolism. Two EH cDNAs were isolated previously by the Roe Lab at North Carolina State University. The first cDNA (TmEH-1) was synthesized by Harris et al. (1999) from the fat body of last stadium day 3 (wandering, L5D3) *Trichoplusia ni* at the exact developmental time of maximum epoxide hydrolase activity. TmEH-1 is 1887 base pairs (bp) in length with a 1389 bp open reading frame (ORF) encoding 463 amino acids. The second cDNA (TmEH-2) was synthesized by Anspaugh (2003) using equal proportions from the digestive system of the last stadium day 1 (L5D1) and day 2 (L5D2) *T. ni* larvae. TmEH-2 cDNA is 2062 bp in length with an ORF of 1389 bp encoding 463 amino acids. To study the difference between these two EHs and their functional role in *T. ni,* an attempt to express the full-length cDNAs was conducted using the InsectSelect^{TM} Glow system (Invitrogen, Carlsbad, CA). In separate experiments, only the ORF of both EHs was used. PCR of genomic DNA from transformed cells, resistance to the antibiotic Zeocin and GFP expression indicated the incorporation of the expression vector into the cell genome. Measurement of EH activity and SDS-PAGE analysis showed that the EH genes were not expressed in transformed cells.

Introduction

Epoxide hydrolases (EHs; EC 3.3.2.3) are members of a diverse group of enzymes that catalyze the hydrolysis of epoxides to the corresponding vicinal diols. Although their amino acid sequence similarity is low (Barth et al., 2004), EHs catalyze the same reaction and belong to the α/β hydrolase fold family (Ollis et al., 1992). EHs have been identified from different living organisms including bacteria, fungi, plants, insects and mammals (Beetham et al., 1995). Barth et al. (2004) analyzed the sequence and the structure of more than 100 EHs available in the GenBank database and classified them into two large superfamilies, the microsomal EH and the cytosolic EH superfamilies. The microsomal EHs (mEHs) contain at the amino-terminus, a membrane insertion signal of ~ 20 amino acids (Friedberg et al., 1994) and have substrate specificity that is different from cytosolic (soluble) EHs (sEHs).

EHs from microorganisms have been of interest because of their potential use in the production of optically pure epoxides and diols (Weijers and de Bont, 1999; Swaving and de Bont, 1998; Steinreiber and Faber, 2000; Zhao et al., 2004). An extensive effort has been undertaken to understand the role of EHs in mammals where five classes of EHs have been recognized: mEH, sEH, hepoxilin A3 hydrolase, leukotriene A4 hydrolase and microsomal cholesterol 5,6-epoxide hydrolase (Fretland and Omiecinski, 2000). The association of some EHs with diseases has been studied, and the presence of inhibitors for these EHs suggests new avenues for disease treatment (Chen et al., 2004; Imig et al., 2002; Zhao et al., 2004).

Little is known about the function of EHs in insects. It is believed that EHs in insects play a role in xenobiotic and juvenile hormone (JH) metabolism. The role of EH in the detoxification of cyclodiene insecticides was first reported in the southern house mosquito (Oonnithan and Miskus, 1964; Tomlin, 1968) and in the housefly (Brooks et al., 1970). Additional studies indicated the presence of epoxide hydrolases in other insects (reviewed by Mullin, 1988). The role of EH in insecticide metabolism was reviewed by Dauterman (1982). Slade et al. (1976) found that EH activity was maximal in the fifth instar southern armyworm coincident with the highest plant consumption rate. Mullin (1988) reviewed the role of EH in detoxifying dietary plant epoxides in herbivorous insects. He showed that EH activity in polyphagous insects is much higher than that in insects that fed on limited parts of the plant and in predaceous insects. Taniai et al. (2003) characterized an EH cDNA from the third instar *Drosophila melanogaster* that is inducible by clofibrate and has no activity towards JH.

Juvenile hormone is important in insect development and reproduction. JH titer is regulated by two main processes, synthesis and degradation (reviewed by Gilbert et al., 2000). The primary pathway for JH metabolism is ester hydrolysis by the highly specific juvenile hormone esterase (JHE) as indicated in many reports (Hammock, 1985; Roe and Venkatesh, 1990; de Kort and Granger, 1996; Gilbert et al., 2000). A second metabolic pathway for JH metabolism is the hydration of the 10, 11-epoxide by epoxide hydrolase. The role of EH in juvenile hormone metabolism was reviewed by Hammock (1985), Roe and Venkatesh (1990) and Gilbert et al. (2000).

EH was purified from *Spodoptera eridania* (Mullin and Wilkinson, 1980a, b) *Manduca sexta* (Touhara and Prestwich, 1993) and *Ctenocephalides felis* (Keiser et al., 2002). EH cDNAs were isolated and characterized from some insects like *M. sexta* (Wojtasek and Prestwish, 1996), *Ctenocephalides felis* (Keiser et al., 2002), *Trichoplusia ni* (Harris et al., 1999; Anspaugh, 2003) and *D. melanogaster* (GenBank database).

Multiple forms of EH in insects was suggested by Mullin (1988). He hypothesized that pH optima, substrate specificity, and tissue and subcellular distribution support the existence of more than one form of EH. This was validated by the sequencing of 3 EH cDNAs from *D. melanogaster* (GenBank database), 3 EH cDNAs from *T. ni* (Harris et al., 1999, Anspaugh, 2003; Khalil, 2004), and 2 EH cDNAs from *Ctenocephalides felis* (Keiser et al., 2002).

The reaction mechanism and the structure of the active site of EH have been extensively studied. Several studies have suggested that the catalytic triad for mEHs consists of Asp/His/Glu residues while that of the sEHs consists of Asp/His/Asp residues (Lacourciere and Armstrong, 1993; Borhan et al., 1995; Linderman et al., 1995; Arand et al., 1996; Roe et al., 1996). Recently, Taniai et al. (2003) identified mEH from *D. melanogaster* (DmEH) with the catalytic triad Asp/His/Asp similar to sEHs. The epoxide hydrolysis reaction goes through a covalently bound ester intermediate formed by attack of the nucleophilic Asp on the least substituted carbon atom of the epoxide ring. The intermediate ester is then hydrolyzed by a water molecule activated by the His/Glu or His/Asp pair. Recent studies on the sEHs suggest that two other tyrosine residues are involved in the formation of the ester intermediate in the first step (Argiriadi et al., 2000;

Rink et al., 2000; Yamada et al., 2000; 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 that are expected to play a role in the first step of the reaction.

To obtain a better understanding of the role of EHs in insects, two EH cDNAs isolated from *T. ni* were subjected to expression in insect cells using the InsectSelect™ Glow system (Invitrogen, Carlsbad, CA). The Full-length cDNAs and the ORFs of both EH cDNAs were used in separate experiments. PCR for genomic DNA from transformed cells, resistance to the antibiotic Zeocin, and GFP expression detection demonstrated the incorporation of the expression vector along with the EH cDNAs into the cell genome. Measurement of EH activity and SDS-PAGE analysis showed that the EH cDNAs were not expressed in the transformed cells.

Materials and Methods

Two different epoxide hydrolase cDNAs have been previously isolated in our lab from the cabbage looper, *Trichoplusia ni*. The first cDNA (TmEH-1) was isolated by Harris et al. (1999) from the fat body of last stadium, day 3 (wandering, L5D3) *T. ni* at the exact developmental time of maximum epoxide hydrolase activity. TmEH-1 is 1887 base pairs (bp) in length with a 1389 bp open reading frame (ORF) encoding 463 amino acids. The second cDNA (TmEH-2) was isolated by Anspaugh (2003) using equal proportions of digestive system from last stadium day 1 (L5D1) and day 2 (L5D2) *T. ni* larvae. TmEH-2 cDNA is 2062 bp in length with a 1389 bp ORF encoding 463 amino acids. The cDNAs were constructed using Zap Express cDNA Synthesis and Gigapack II

Gold Cloning kits (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Finally, cDNAs were cloned into pBK-CMV cloning and expression vector (Fig. 1, Stratagene) producing pSVH03 (carrying TmEH-1) and pDDA01 (carrying TmEH-2). For protein expression, The InsectSelect^{TM} Glow system (Invitrogen, Carlsbad, CA) was used.

Subcloning of full-length EH cDNAs in the pIZT/V5-His

The plasmids pSVH03 and pDDA01 (Fig. 1) were digested by *Not*I and *Sac*I restriction enzymes (Promega, Madison, WI) to release the EH cDNAs. Also, pIZT/V5-His (Fig. 2) was digested with the same enzymes. Digested plasmids were separated by agarose gel electrophoresis. EH cDNAs and digested pIZT/V5-His were eluted from the gel using the QIAquick gel extraction kit (QIAGEN, Valencia, CA). TmEH1 cDNA was ligated to digested pIZT/V5-His using T4-ligase (Promega) producing pSK1 (Fig. 2). Also, TmEH-2 was ligated to digested pIZT/V5-His producing pSK2 (Fig. 2). Standard molecular biology procedures were according to Sambrook et al. (1989).

Selecting stable cell lines

Transfection and selection of stable Sf9 cells were conducted according to the InsectSelect^{TM} Glow system (Invitrogen). In two separate experiments, Sf9 cells were transfected with the recombinant plasmids pSK1 and pSK2. Sf9 cells were maintained on Grace's medium containing Zeocin, 400 μg /ml medium.

EH assay

Five ml of non-transformed and transformed cell cultures were harvested and cells were collected by centrifugation at 1000*g* at 4ºC for 10 min. Cell pellets were

homogenized in 1 ml ice-cold sodium phosphate buffer (0.1M, pH 7.4) for 1 min at speed 4 using a Polytron PT10/35 homogenizer (Brinkmann Instruments, Westbury, NY). Homogenates were used directly for EH assay. The assay was carried out as described by Share and Roe (1988). The substrate used was a mixture of [^{3}H]-racemic JH III (12 Ci/mmol, tritiated at C10; PerkinElmer Life Sciences, Boston, MA) and unlabeled racemic JH III (Sigma, Milwaukee, WI). The final concentration of JH III substrate was $5x10^{-4}$ M in absolute ethanol having ~ 8000 cpm/µl. Cell homogenates (100µl) were incubated first with 1µl of 0.01M OTFP at 30 ºC for 10 min to inhibit JH esterase activity in the Sf9 cells. JH III substrate (1µl) was then added to each reaction and tubes incubated at 30 ºC for 15 min. The reaction was quenched with methanol (300 µl) and isooctane (250 µl). Tubes were vigorously vortexed and centrifuged at 1000 *g* for 5 min to separate phases. Aliquots of 100µl each from the top and bottom phases were analyzed by liquid scintillation. Protein concentrations of different cell homogenate samples were determined by the Bio-Rad assay (Bio-Rad Laboratories, 1977), using bovine serum albumin (Fraction V; Fisher Scientific) as a standard.

SDS-PAGE

SDS-PAGE was conducted to test the EH protein expression. Fifteen µg protein in 5µl phosphate buffer from each cell homogenate were mixed with an equal volume of 2x sample preparation buffer containing 2-mercaptoethanol (Zaxis, Hudson, OH) and heated to 95°C for 5 min. Proteins were separated by electrophoresis on a 10x10 cm, 4 (stacking) -12 % (separating) polyacrylamide Tris-Glycine Gel (Zaxis) using the Zaxis system 2000EP and 1x Tris-Glycine running buffer (Laemmli, 1970). The gel was run at

150 V until the dye markers were approximately 1 cm from the bottom of the gel. The proteins were visualized by staining with Coomassie blue (0.05 % Coomassie blue in 10% acetic acid and 50% ethanol) and destaining in 7% acetic acid and 5 % methanol.

PCR

Genomic DNA was isolated from Sf9 cells using the QIAamp DNA minikit (QIAGEN). PCR was conducted using 200 ng genomic DNA, 50 pmol of both OpIE2 Forward (5' CGCAACGATCTGGTAAACAC 3') and OpIE2 Reverse (5' GACAATACAAACTAAGATTTAGTCAG 3') primers, 1x reaction buffer, 200μM each dNTP, and 2 units *Taq* DNA Polymerase (Promega) in a total volume of 50 μl. Both OpIE2 Forward and OpIE2 Reverse primers were supplied in the InsectSelect^{TM} Glow system (Invitrogen) and correspond to nucleotides 515 to 534 and 766 to 741 of the pIZT/V5-His plasmid, respectively. PCR conditions were 95°C for 5 min followed by 35 cycles each consisting of 95°C for 1 min, 60°C for 1 min and 72°C for 2 min. Finally, an extension of 72°C for 7 min was done after the last cycle. Five μl of each reaction were analyzed using agarose gel electrophoresis.

GFP expression

GFP expression was detected using a Leica DMLB microscope (Leica, Deerfield, IL). Pictures were taken using a Hamamatsu ORCA-ER camera (Hamamtsu, Bridgewater, NJ) in the Cellular and Molecular Imaging Facility at North Carolina State University. GFP excitation was achieved with a FITC filter 480/30 which covers the second excitation zone (478nm) of the GFP. Emission was detected with a FITC filter 535/40 which covers the emission zone (507nm) of the GFP.

Amplification and subcloning of EH ORFs in the expression vector

Expression of the full-length cDNAs of TmEH-1 and TmEH-2 in Sf9 cells was not successful. An alternative approach to achieve expression was to use the ORF of both EHs. Theses studies were conducted with both Sf9 and High Five cells to examine the effect of the 5' and 3' untranslated regions and the importance of cell type, respectively, on EH expression. To amplify the ORF of TmEH-1, two primers were designed, FBEHFor (5' TT**GAGCTC**TTT*AATATGG*GTCGCCTCT TA 3') and FBEHRev (5' AA**GAATTC**AAATCAGTCTTCTCGTTATT 3') that correspond to nucleotides 76 to 96 and nucleotides 1476 to 1456 of TmEH-1, respectively. To amplify the ORF of TmEH-2, two additional primers were designed, GUTEHFor (5' TT**GAGCTC**TTT*AATATG*GCCCGTCTCCT 3') and GUTEHRev (5' CC**GAATTC**AAATCAGTCTTGACATTCTT 3') that correspond to nucleotides 32 to 51 and nucleotides 1425 to 1406 of TmEH-2, respectively. Both FBEHFor and GUTEHFor contain the *Sac*I restriction site (shown in bold), the native start codon and Kozak sequence of both EHs (shown in italics). Also, both FBEHRev and GUTEHRev contain the *EcoR*I restriction site (shown in bold). FBEHRev and GUTEHRev were designed to avoid the native stop codon of both EHs, to be in frame with the 6xHis tag from the expression vector, and to utilize the stop codon of the latter. Primers were tested for hairpin and dimer formation using the OLIGO 4 program (MBI, West Cascade, CO) and were synthesized by Sigma-Genosys (Woodlands, TX). PCR amplification for the EH ORFs were carried out as explained above except for using AccuPrime *Taq* DNA

polymerase High Fidelity (Invitrogen), extension at 68 °C for 3 min in each cycle (instead of 72 °C for 2 min) and a final extension step at 68 °C for 7 min. PCR amplification products were purified using the QIAquick PCR Purification Kit (QIAGEN) and digested with *SacI* and *EcoR*I (Promega). pIZT/V5-His was also digested with the same enzymes. Digestion products were analyzed by agarose gel electrophoresis. Digested ORFs of TmEH-1, TmEH-2 and digested pIZT/V5-His were eluted from the gels and ligated as described earlier. Expression of the ORFs was carried out using both Sf9 and High Five insect cells (Invitrogen). PCR of the genomic DNA from cells, EH assay and SDS-PAGE for cell homogenates were also conducted as explained earlier.

Results and Discussion

Expression of the full-length EH cDNAs in Sf9 cells

Subcloning of the full-length EH cDNAs in pIZT/V5-His

Both EH cDNAs were digested from the plasmids, pSVH03 and pDDA01 (Fig. 1), using *Not*I and *Sac*I restriction enzymes. TmEH-1 and TmEH-2 cDNAs were cloned into pIZT/V5-His (Fig. 2) digested with the same enzymes. pIZT/V5-His contains the Zeocin-GFP fusion gene under the control of the OpIE1 promoter isolated from the baculovirus *orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (*Op*MNPV). The Zeocin-GFP fusion gene allows the selection of transformed cells on Zeocin antibiotic. It also allows the verification of transformation through detection of the GFP when illuminated with UV light of the right wavelength. The resulting expression plasmids, pSK1 and pSK2 (Fig. 2), contain the EH cDNAs under the control of the OpIE2 promoter isolated

from the same virus. *Op*MNPV natural host is the Douglas fir tussock moth; however, OpIE1 and OpIE2 promoters allow protein expression in both Sf9 and High Five cells (Invitrogen). pSK1 (carrying TmEH-1) and pSK2 (carrying TmEH-2) were digested with *Not*I and *Sac*I to verify the inserts (Fig. 3). Fig. 3 shows the results of the restriction digestion of different EH cDNAs constructs using *Not*I and *Sac*I restriction enzymes. Lane 1 shows the results of digestion of pSVH03 where two bands of ~ 4500 bp (pBK-CMV plasmid, used in the cloning of the EH cDNAs), and of ~ 1850 bp (TmEH-1) were obtained. Lane 2 shows the results of the digestion of pSK1 where two bands of ~ 3300 bp (pIZT/V5-His), and of ~ 1850bp (TmEH-1) were obtained. TmEH-1 was obtained at both digestion and migrated at the same rate (~1850 bp). Similarly, lanes 3 and 4 show the results of the digestion of pDDA01 and pSK2, respectively. In both cases, TmEH-2 was obtained and migrated at the expected rate (~2000bp). After cloning in the expression vector, TmEH-1 and TmEH-2 cDNAs were sequenced using OpIE2 forward primer. Analysis of the resulting sequences indicated that cDNAs were cloned under the OpIE2 promoter in the right orientation. It also showed the EH cDNA native Kozak sequences, the ATG start codon, and ~ 600 bases of sequence without any mutations.

Selecting stable cell lines

After verification of the right sequences, Sf9 cells were transfected with pSK1 and pSK2 in two separate experiments. Sf9 cells were established on Grace's medium containing 400 μg Zeocin /ml medium. Zeocin was used because the expression vectors contain the Zeocin-GFP fusion gene which allows transfected cells to grow on Zeocin containing medium. Non transformed cells were also tested at the same Zeocin

concentration. Non transformed cells were killed after 6 days while transformed cells survived. Also, transformed cells survived with Zeocin concentrations up to 1000 μg Zeocin/ml medium. This concentration killed non transformed Sf9 cells after 24h. The survival of transformed Sf9 cells on high concentrations of Zeocin indicated that cells acquired the Zeocin resistant gene and therefore must contain the expression vectors.

PCR

Genomic DNA from SF9 non transformed and transformed cells was isolated and analyzed by PCR using the two primers, OpIE2 forward and OpIE2 reverse. The expected amplification products were obtained in all cases.

Fig. 4 shows the amplification product from Sf9 cells transformed with pIZT/V5-His in lane 3 where a product of ~ 250 bp was obtained. This amplification product corresponds to the nucleotides 514 to 766 of the pIZT/V5-His plasmid. Lanes 1 and 2 show amplification products from Sf9 cells transformed with pSK1 and pSK2 carrying the full length of TmEH-1 and TmEH-2, respectively. The expected PCR product was obtained in both cases. An approximate 2050 bp amplification product was obtained in lane 1 corresponding to the TmEH-1 cDNA plus ~ 200bp flanking the cloning site of the expression vector. Also, an amplification product of ~ 2200 bp was obtained in lane 2. corresponding to TmEH-2 cDNA plus the 200bp from the expression vector. PCR from non transformed cells did not give any product (lane 4). The PCR results showed that the expression vectors along with the full-length EH cDNAs were incorporated in the insect cell genome.

GFP expression

GFP expression is shown in Fig. 4. Panels A, B, C and D show Sf9 cells as seen by light microscopy while panels a, b, c and d show Sf9 cells under UV light. As expected, non transformed cells (Fig. 5 a) did not show any fluorescent emission when illuminated with UV light, while transformed cells showed fluorescent emission under UV light (b, c and d). These results showed that there is no background fluorescent emission in Sf9 cells and the fluorescent light detected is a result of the GFP expressed from the transformation vectors. Cells transformed with pIZT/V5-His, pSK1, and pSK2 (Fig 9 b, c and d, respectively) demonstrated different levels of fluorescent emission indicating different levels of GFP expression.

SDS-PAGE

Crude homogenates from non-transformed and transformed insect cells were analyzed using SDS-PAGE and stained with Coomassie blue to determine if a new protein is detected in the homogenates of transformed cells (Fig. 6). The expected molecular weight of the EH protein is about 45 K (Harris et al., 1999). Coomassie blue stain did not show a new protein of the expected molecular weight (45 K). Analyzing the protein pattern of non-transformed Sf9 (Fig. 6, lane 1) and cells transformed with, pIZT/V5-His (lane 2), pSK1 (lane 3), and pSK2 (lane 4) did not show a new protein in lanes 3 and 4. These results indicated that EH protein was not expressed at all or expressed in undetectable levels.

EH activity assay

No differences were found in JH III EH activity between homogenates from transformed and non-transformed Sf9 cells (t-test, $P<0.05$), activity ranged between 0.003 and 0.005 nmol JH III/min/mg protein. As mentioned earlier, also the EH protein was not detectable upon analysis by SDS-PAGE and Coomassie blue staining.

Although sequence analysis, PCR results, resistance to Zeocin, and GFP expression indicated that the expression vectors were integrated in the cell genome, the active enzyme was not obtained. This might be because of the interference of the regulatory elements included in the 3' and 5'untranslated regions of the cDNAs with the regulatory elements included in the expression vector. Similar reasons were mentioned by O'Reilly et al. (1992) for baculovirus expression systems.

Expression of the ORF of EH cDNAs in Sf9 and High Five cells

Amplification and subcloning of EH ORFs in the expression vector

TmEH-1 ORF was amplified from pSVH03 using the PCR primers, FBEHFor and FBEHRev. Also, TmEH-2 ORF was amplified from pDDA01 using GUTEHFor and GUTEHRev. The ORF of both EHs is 1389bp. The amplified ORFs of both EHs are shown in Fig. 7. lane 1 and 2. The PCR amplification products were then subcloned into the pIZT/V5-His and the resulting constructs (pSK11 and pSK22, Fig. 8) were digested using *Sac*I and *Eco*RI (Fig. 9) to verify the inserts. Lane 1 shows the uncut pSK11 while lane 2 shows the pSK11 digested with both enzymes. The smaller fragment (~1500 bp) represents the TmEH-1 ORF and the larger fragment (~ 3300 bp) represents the expression plasmid, pIZT/V5-His. Similar results were obtained from the digestion of

pSK22 carrying the ORF of TmEH-2. Undigested pSK22 is shown in Fig. 9 (lane 3) and pSK22 digested with *Sac*I and *EcoR*I is shown in lane 4. Sequences of pSK11 and pSK22 were verified using the OpIE2 forward primer. The sequence results showed the start codon and the native Kozak sequence of TmEH ORFs followed by ~ 600 bases without apparent mutations.

Selecting stable cell lines

After transfection with the recombinant plasmids (pSK11 and pSK22), Sf9 cells were established on Grace's medium containing 400 μg Zeocin /ml medium. High Five cells were established on Express Five SFM medium containing 600 μg Zeocin/ml medium. Transformed High Five cells survived this concentration while non-transformed cells were killed after 5 days. The survival of transformed Sf9 and High Five cells on high concentrations of Zeocin indicates that both cells acquired the Zeocin resistance and contained the expression vectors.

PCR

Genomic DNA from Sf9 non-transformed and transformed cells was isolated and subjected to PCR using the two primers, OpIE2 forward and OpIE2 reverse. High Five cells genomes were also purified and analyzed by PCR using the same primers. The expected amplification products were obtained in all cases.

Fig. 10 shows the amplification product from Sf9 cells transformed with pSK11 and pSK22 in lane 3 and 4, respectively. A product of ~ 1600 bp was obtained in each case. This product corresponds to the EH ORF and 200bp from the expression vector.. Lanes 1

and 2 show no amplification products obtained upon the amplification of the genomic DNA isolated from Sf9 non-transformed cells

Similar results were obtained when High Five cells were transformed with pSK11 and pSK22. Fig. 11 shows the PCR amplification product of High five cells transformed with pSK11 and pSK22 carrying the ORFs of TmEH-1 and TmEH-2, respectively. The expected amplification products (~ 1600 bp) were obtained when cells transformed with pSK11 (lane 3) and pSK22 (lane 4). PCR results indicate that the expression vectors along with the EH TmEH ORFs were incorporated in the insect cell genome.

SDS-PAGE

Crude homogenates from non-transformed and transformed insect cells were analyzed using SDS-PAGE. Coomassie blue staining of separated proteins from Sf9 cells (Fig. 12) did not show new proteins in the homogenate of transformed cells. These results are similar to that obtained from the expression of the full-length cDNAs in Sf9 cells.

SDS-PAGE analysis of homogenate from High Five cells showed a new protein band at ~ 50 K in the homogenates of transformed cells (indicated by arrow in figure 12). This 50 K protein might correspond to the EH expressed protein.

EH activity assay

In Sf9 cells, EH activity was almost undetectable in transformed and non-transformed cells. Also, EH protein was not detected upon Coomassie blue staining of the SDS-PAGE. These results, along with results from the full-length expression, indicate that Sf9 cells may not be the right choice for EH expression using the InsectSelect Glow System.

Although a band of ~ 50 K was detectable in the homogenate of transformed High Five cells, the EH activity was the same in transformed and non-transformed cells (0.134±0.001 (±1SEM), 0.137±0.013 and 0.1190±0.004 nmol JH III/min/mg protein for non-transformed, pSK11 and pSK22 transformed High Five cells, respectively) indicating that the expressed protein does not have the EH activity.

In case of expression of the full length cDNAs, we mentioned that the interference of regulatory elements from the cDNA with regulatory elements included in the transformation vector might be the reason for the failure of the expression. In case of the expression of the ORFs, regulatory elements were removed from the 5' and the 3' regions of the cDNAs and the native Kozak sequence was included as recommended by the manufacturer. The Kozak sequence is necessary for vertebrate mRNA expression (Kozak, 1987). However, in Sf9 cells, the EH protein was not detected on SDS-PAGE and no enhanced EH activity was found. For High Five cells, a new protein of the expected molecular size was detected on SDS-PAGE but this was not positively correlated with an increase in the EH activity. Similar results were obtained when Keiser et al. (2002) expressed the ORF of the cat flea, *Ctenocephalides felis,* EH1 (CfEH1) in *E. coli* where they obtained the expected EH protein but they did not mention whether it was active or not, apparently indicating that the expressed EH protein was not active. Keiser et al. (2002) removed the regulatory elements and also the first 22 amino acids (the membrane insertion signal) from the cDNA used in the expression.

The only successful expression of insect mEH was achieved using the baculovirus expression system. Debernard et al. (1988) and Taniai et al. (2003) expressed the mEH

ORFs of *Manduca sexta* and *Drosophila melanogaster*, respectively. Also, Harris et al. (1999) expressed the full-length cDNA of TmEH-1 from *T. ni* using the baculovirus expression system.

The stable non-lytic system (InsectSelact Glow) was chosen in this study because once the transformed cells are obtained, there is no need for transfection in each cell passage as the case in baculovirus expression system. Also, this system does not kill the insect cells and gives the viable environment for the expressed enzymes needed for the enzymatic activity but baculovirus destroys the internal cellular structures and eventually kills the cells.

In summary, the expression of both the full-length cDNA and the ORF of TmEH-1 and TmEH-2 using the InsectSelect Glow System was not successful. Although sequence analysis, resistance to Zeocin, GFP expression, and PCR results showed that the expression vectors were present in the cell genome without introduced mutations, the active EH protein was not obtained as indicated by measurement of the EH activity.

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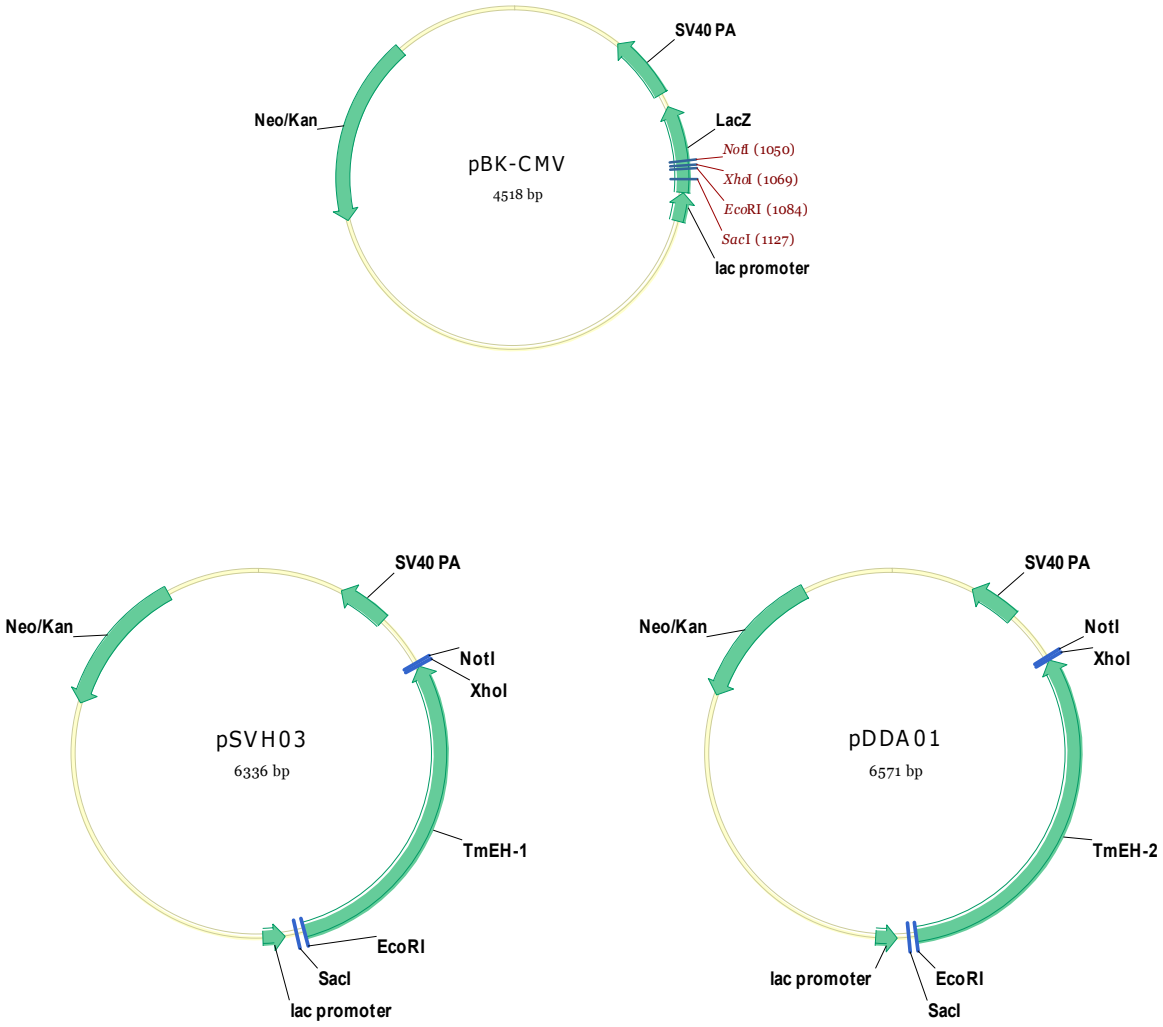

Fig. 3.1. pBK-CMV cloning and expression vector. pSVH03 and pDDA01 resulted from the cloning of TmEH-1 and TmEH-2, respectively, into pBK-CMV.

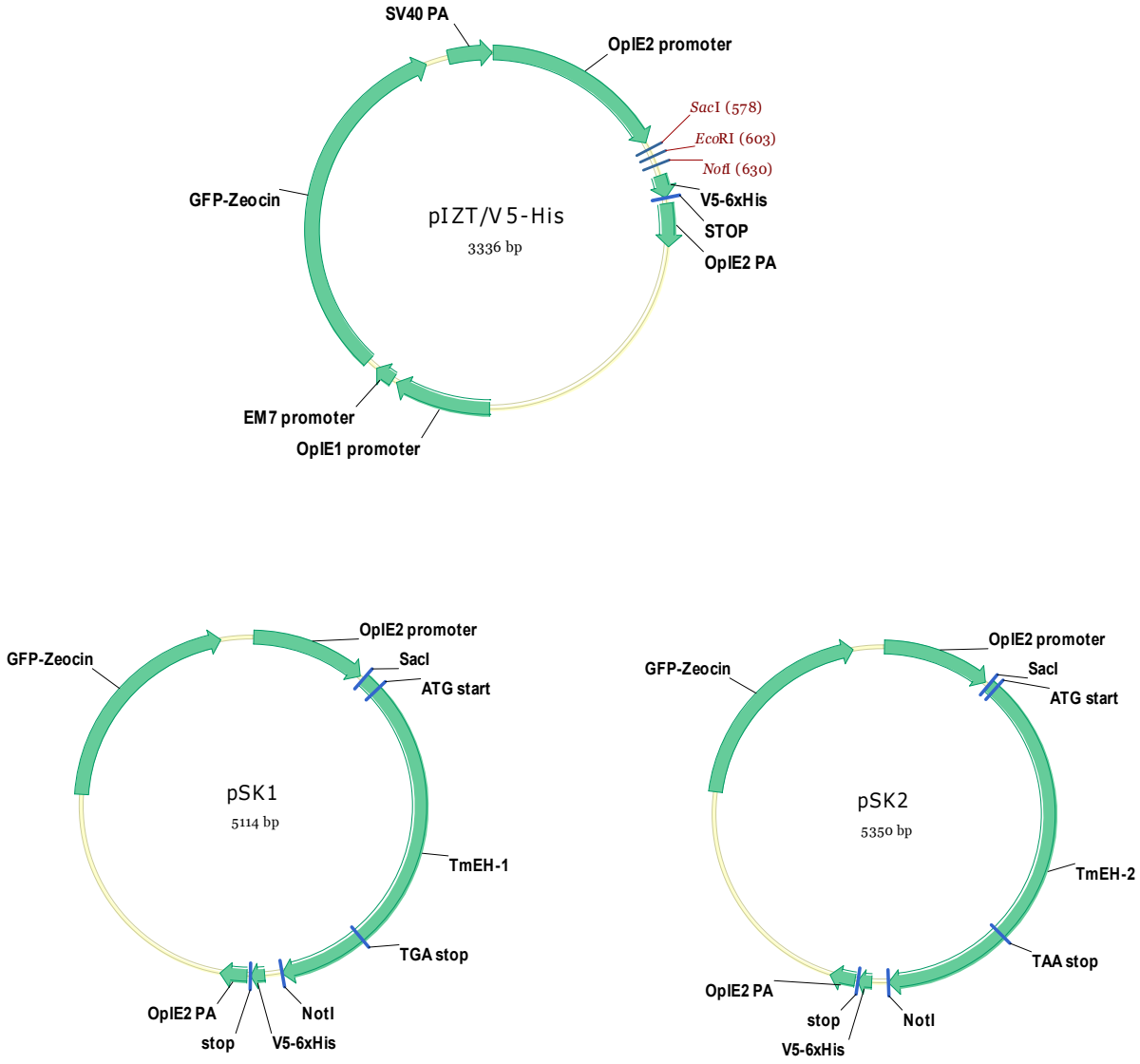

Fig. 3.2. pIZT/V5-His expression vector. pSK1 and pSK2 resulted from the cloning of TmEH-1 and TmEH-2, respectively, into pIZT/V5-His.

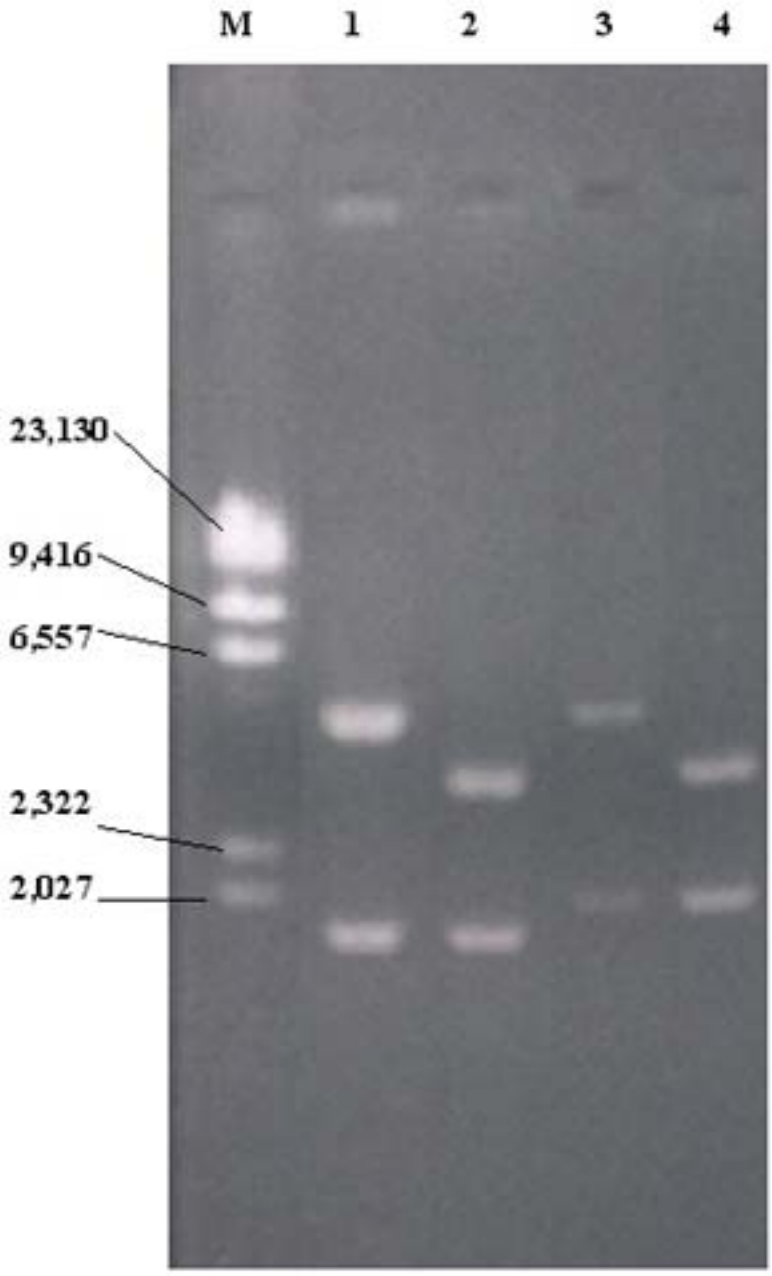

Fig. 3.3. Restriction digestion of different EH cDNA constructs using *Not*I and *Sac*I restriction enzymes. M, λ DNA/*Hind*III molecular size markers; lane 1, pSVH03; lane 2, pSK1; lane 3, pDDA01; and lane 4, pSK2. Digested constructs were analyzed using a 1% agarose gel.

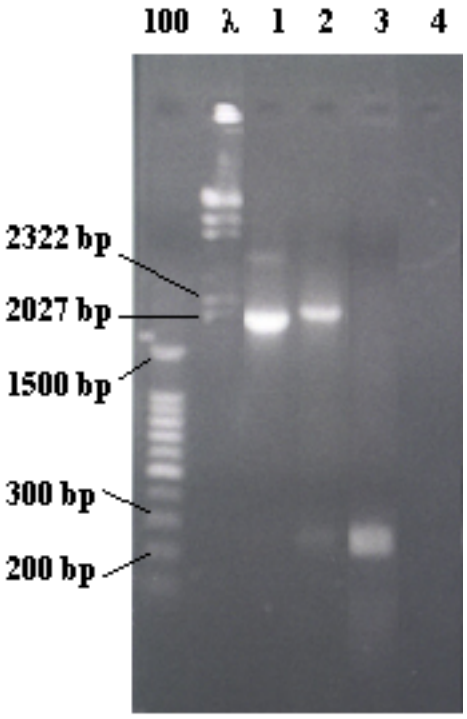

Fig. 3.4. PCR amplification products for non-transformed and Sf9 cells transformed with pSK1, pSK2 and pIZT/V5-His using OpIE2 forward and OpIE2 reverse primers. 100: 100 bp DNA ladder, λ: λ DNA/*Hind*III molecular size markers. PCR amplification product using genomic DNA from Sf9 cells transformed with: pSK1 (lane 1), pSK2 (lane 2), pIZT/V5-His (lane 3), and non transformed cells (lane 4).

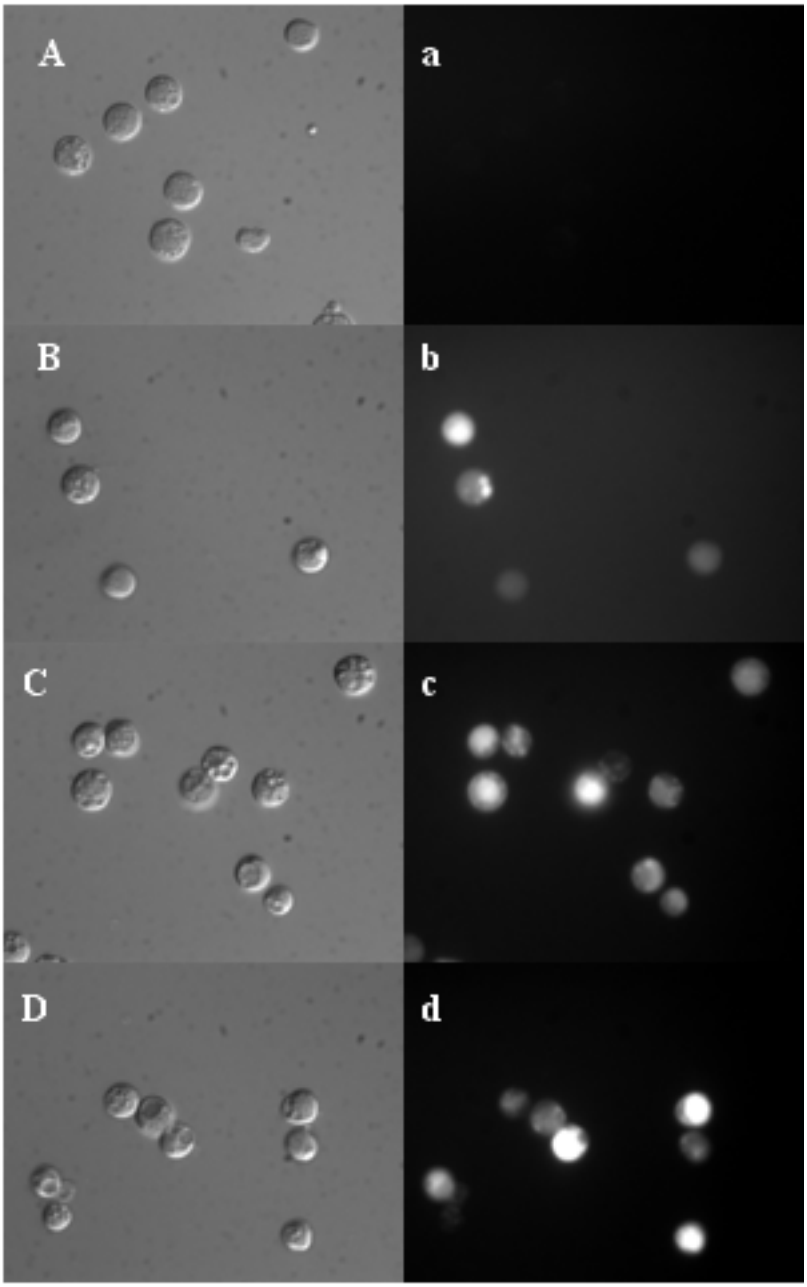

Fig. 3.5. Expression of GFP in Sf9 cells. A, B, C and D are cells as seen by light microscopy. a, b, c and d are cells after illumination with UV light. A and a, Sf9 cells non-transformed; B and b, Sf9 cells transformed with pIZT/V5-His; C and c, Sf9 cells transformed with pSK1; D and d, Sf9 cells transformed with pSK2.

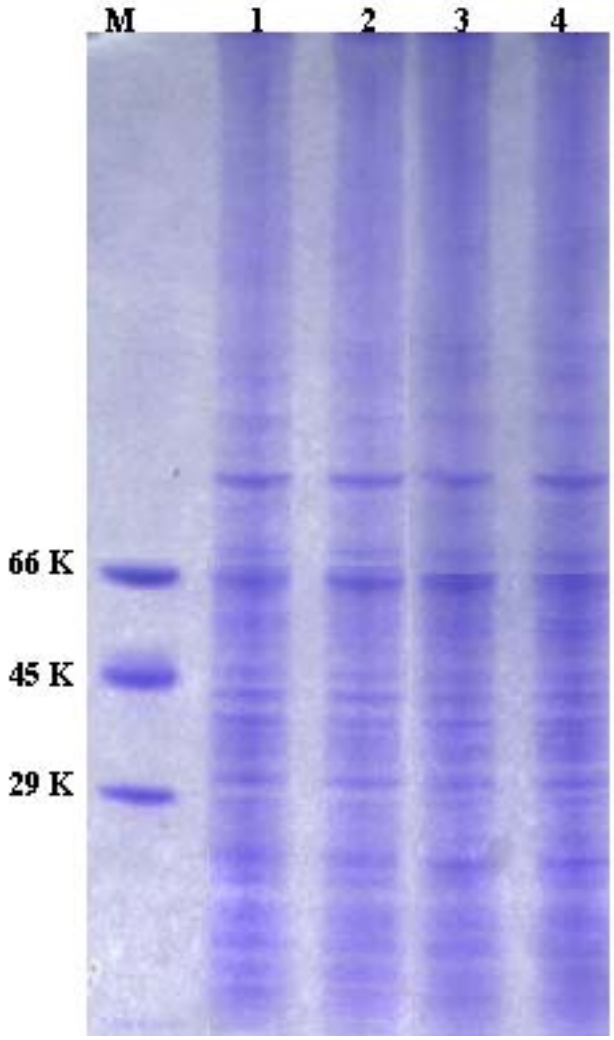

Fig. 3.6. SDS-PAGE of crude homogenates of Sf9 cells non-transformed (lane 1), transformed with pIZT/V5-His (lane 2), transformed with pSK1 (lane 3) and transformed with pSK2 (lane 4). M, protein molecular weight markers.

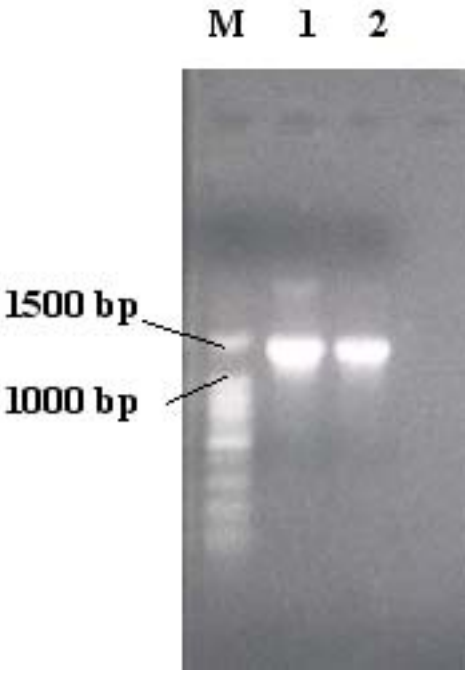

Fig. 3.7. PCR amplification of ORFs of TmEH-1 and TmEH-2. ORfs were amplified from pSVH03 (Tm-EH1) and pDDA01(Tm-EH2) using the FBEHFor/FBEHRev and GUTEHFor/GUTEHRev, respectively. M, 100 bp DNA ladder. Amplified ORF of TmEH-1 is shown in lane 1 while amplified ORF of Tm-EH2 is shown in lane 2.

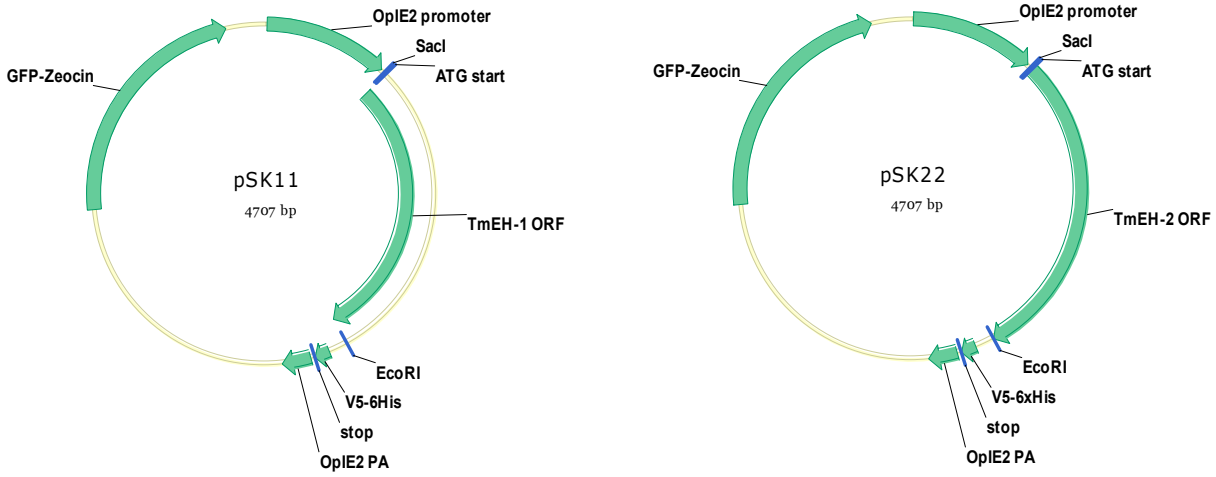

Fig. 3.8. pSK11 and pSK22 resulted from the cloning of the ORF of TmEH-1 and TmEH-2, respectively, into pIZT/V5-His.

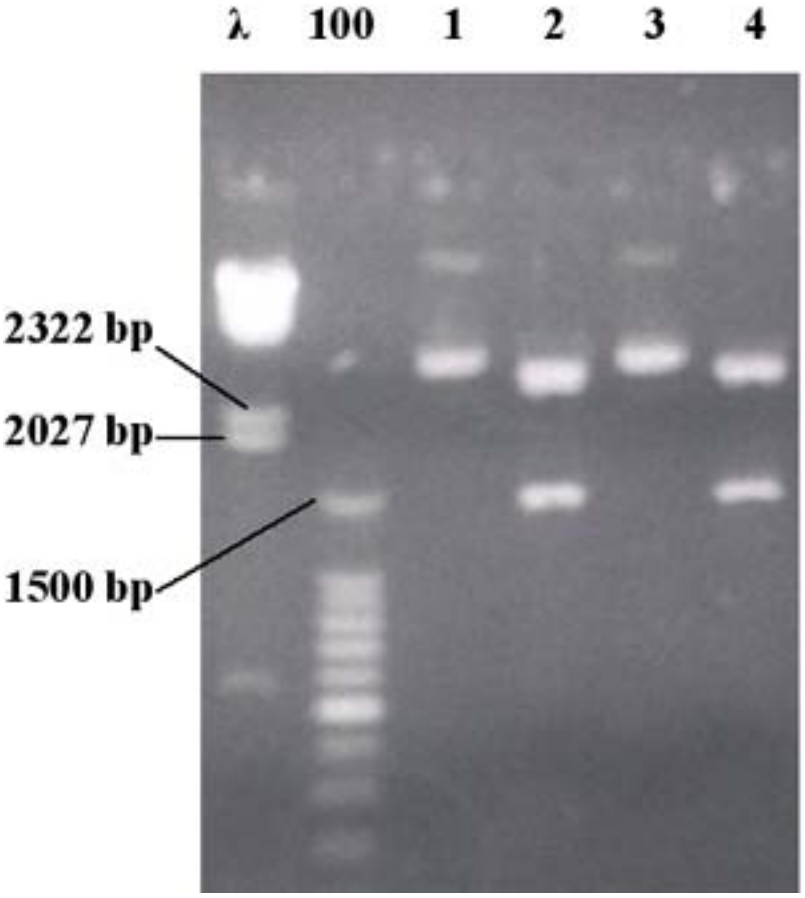

Fig. 3.9. Restriction digestion of pSK11 and pSK22 constructs using the *EcoR*I and *Sac*I restriction enzymes. λ, λ DNA/*Hind*III molecular size markers; 100, 100 bp DNA ladder; lane 1 undigested pSK11; lane 2, digested pSK11; lane 3, undigested pSK22; and lane 4, digested pSK22. Undigested and digested constructs were analyzed using a 1% agarose gel

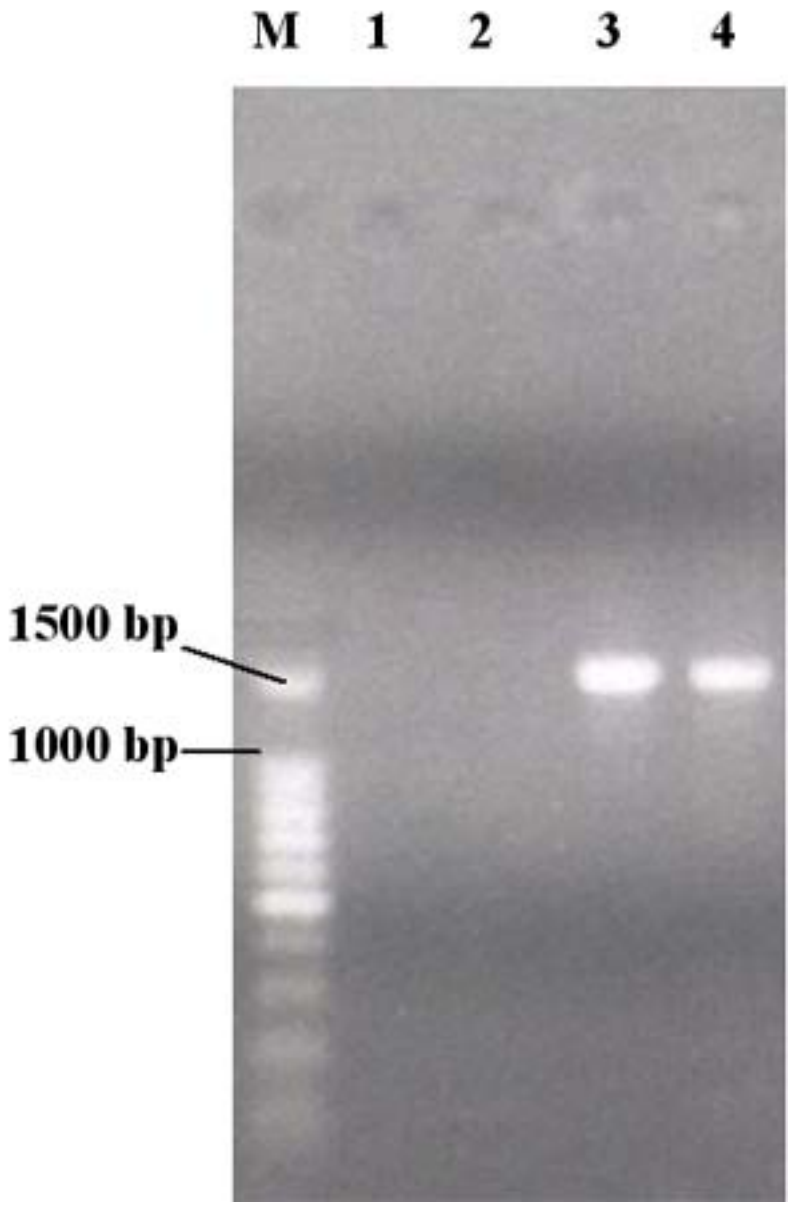

Fig. 3.10. PCR amplification products for genomic DNA isolated from non-transformed and Sf9 cells transformed with pSK11 and pSK22 using OpIE2 forward and OpIE2 reverse primers. M, 100 bp DNA ladder; lanes 1 and 2, PCR results using genomic DNA from Sf9 non-transformed cells; lane 3: PCR product from Sf9 cells transformed with pSK11; lane 4, PCR product from Sf9 cells transformed with pSK22.

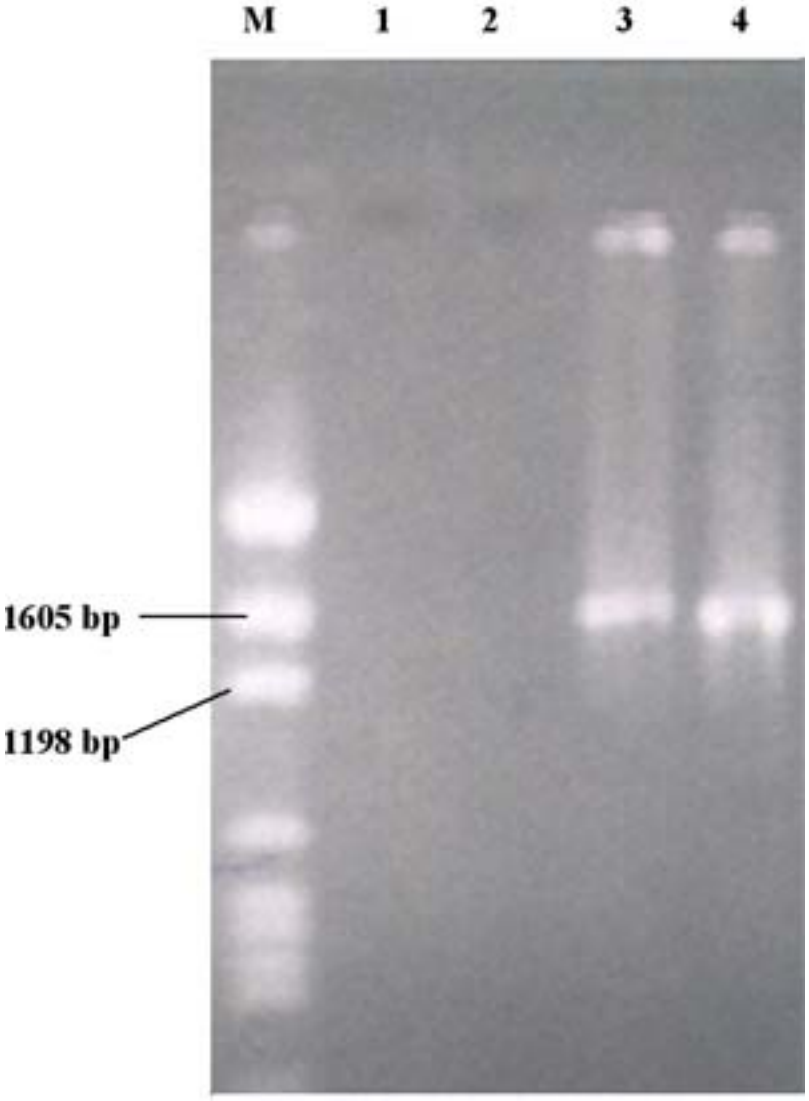

Fig. 3.11. PCR amplification products for genomic DNA isolated from non-transformed and High Five cells transformed with pSK11 and pSK22 using OpIE2 forward and OpIE2 reverse primers. M, pGEM DNA markers; lanes 1 and 2, PCR results using genomic DNA from High Five non-transformed cells; lane 3, PCR product from High Five cells transformed with pSK11; lane 4, PCR product from High Five cells transformed with pSK22.

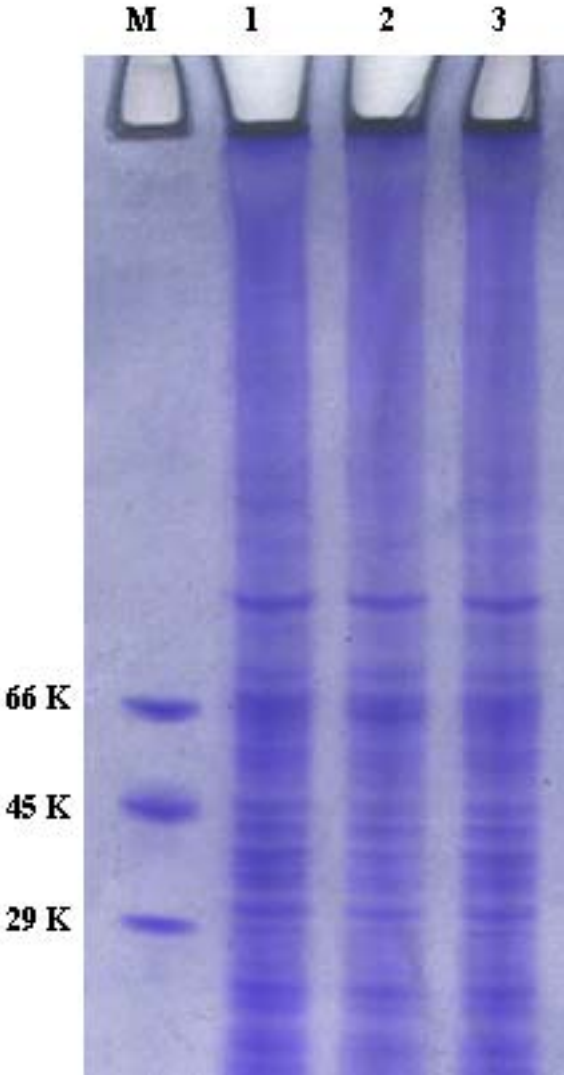

Fig. 3.12. SDS-PAGE of crude homogenates of Sf9 cells non-transformed (lane 1), transformed with pSK11 (lane 2) and transformed with pSK22 (lane 3). M, protein molecular weight markers.

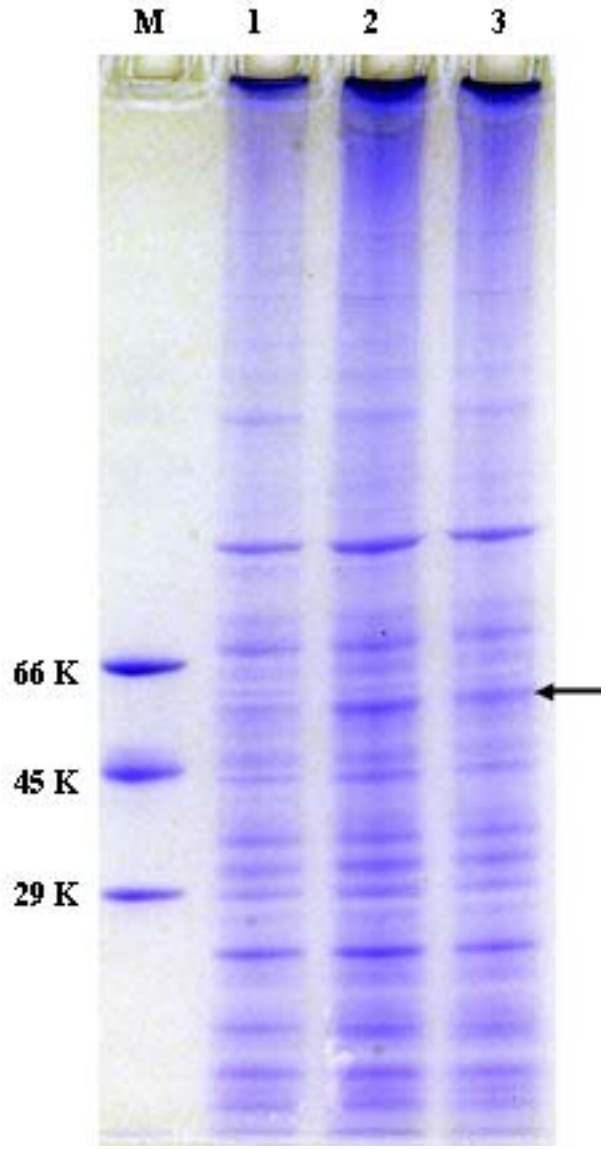

Fig. 3.13. SDS-PAGE of crude homogenates of High Five cells non-transformed (lane 1), transformed with pSK11 (lane 2) and transformed with pSK22 (lane 3). M, protein molecular weight markers. The arrow indicates the new protein in lanes 2 and 3.

APPENDIX

DEVELOPMENT OF A LABORATORY STRAIN OF THE TOBACCO BUDWORM RESISTANT TO DENIM® AND A FIELD KIT FOR RESISTANCE MONITORING

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Abstract

A laboratory strain of the tobacco budworm, *Heliothis virescens,* was selected every generation by larval dip using Denim® (emamectin benzoate). A moderate level of resistance was detected after selection for seven successive generations. The LC_{50} for the parental strain by dip was 0.8 PPM while that for the selected strain (generation 8) was 6.1 PPM, a resistance ratio of 7.6-fold. Using this resistant strain, we developed a feeding disruption assay for the diagnosis of Denim® resistance in neonates of the tobacco budworm. The presence of blue feces on a white background is a marker for resistance. The kit is in the commercialization phase of development.

Introduction

Emamectin benzoate is a semisynthetic derivative of avermectin that has potent acaricidal, insecticidal, and nematicidal activity (Putter et al., 1981). Avermectins are produced in the fermentation process of the actinomycete, *Streptomyces avermitilis*, which was first isolated from a soil sample collected from Japan (Burg et al., 1979). Avermectins act on different types of chloride-gated channels regulating skeletal muscles in insects and other invertebrates (Arena, 1994). Avermectins have little or no cross resistance with other chemical classes (Clark et al., 1994), have very little effect on non target organisms and beneficials (Lasota and Dybas, 1991), neither persist nor accumulate in the environment and are used at very low rates in the field (Jansson and Dybas, 1998). Emamectin benzoate was developed to act specifically on lepidopteran pests and has LC_{50}s less than 1 PPM (Jansson et al., 1996). Our goal was to evaluate the

potential for insect resistance development to Denim using an important economic pest of cotton and other crops, the tobacco budworm. We also introduce in this paper a new diagnostic kit for monitoring larval resistance to Denim that is based on the combination of three technologies--feeding disruption as measured by fecal production, hydrateable meal pads and a new device (Bailey et al., 1998; Roe et al., 1999; Roe et al., 2000a,b; Roe et al., 2002).

Materials and Methods

Insects

Tobacco budworm larvae were reared individually in 30 ml plastic cups (Solo Cup Co., Urbana, IL) on standard artificial diet (Burton 1970) at 27±1ºC with a 14:10 (light:dark) cycle and 50-60% relative humidity. Adults were fed a 20% sucrose solution and kept at the same conditions.

Selection

Late third instars (30±5 mg) of the tobacco budworm were used for selection. Treatment was carried out by larval dip in the appropriate Denim® solution (0.16% EC) made in distilled water. Larvae were dipped in the solution using soft forceps, placed on a paper towel to dry at room temperature (1 min), and then returned to the rearing cup with diet where they were allowed to develop to the pupal stage under standard rearing conditions. The pupae were removed from the diet and allowed to mate in mass and lay eggs. We treated 1000-1200 larvae per generation and obtained 200-500 pupae from these larvae. The treatment concentration was increased each generation to keep mortality between 60-70%.

LC_{50}s

LC_{50}s for both the susceptible and 8th generation of selection were obtained by larval dip (the same technique used for selection). The insects used to estimate the LC_{50} were not selected in the 8th generation. Larval mortality was recorded 12 days after treatment. Two replicates from the susceptible strain and three replicates from the 8th generation selected strain were used to estimate the LC_{50}. Five doses and 25 larvae (third instars) per dose were used for each replicate. Abbot's correction (Abbott, 1925) was applied to all data. Median lethal doses were estimated plots of probit mortality versus log dose (Finney, 1971) using the method of least squares and inverse predictions of 95% fiducial ranges (Sokal and Rohlf, 1995). Calculations were made in Microsoft Excel spreadsheets (Microsoft, 1997).

Feeding Disruption Assay

Feeding disruption assays were conducted on parental (Denim susceptible) and selected (8th generation) budworms using the new Agdia® resistance assay plates (Figure 1). Each plate contains 16 wells in a 4x4 format. The dark circle is a hydrateable meal pad with a blue indicator dye used to monitor feeding. The meal pads extend below the well and are open below the well to the outside. Hydration is maintained via a wetted surface below the wells. For a detail protocol on the use of these plates, refer to Roe et al. (2002). The dry meal pads were rehydrated from the top using 100 µl of distilled water (control) or the appropriate concentration of Denim® in distilled water for 30 min before adding insects to the well. Excess liquid was removed using Q-tip cotton swaps. Neonates (one larva for each well) were transferred to the diet using a camel hair brush,

wells were sealed using a semipermeable, transparent tape, and the plates placed on the surface of a wet paper towel in a sealed plastic container. Plates were incubated at standard rearing conditions. The number of blue fecal pellets produced per well was recorded 24h after the addition of the insects. The pellets were counted using a dissecting microscope. Fecal production is a measure of the susceptibility of the insect to Denim in the meal pad. Larval mortality was also determined. Insects were considered dead if they did not respond to touch by a blunt probe.

Results and Discussion

Selection History

The selection history is summarized in Figure 2. The first round of selection was 1 PPM of Denim® and produced 67% mortality. At this selection dose in the second generation, mortality was reduced to 47.5%. For generations 3-8, we had to increase the selection dose every generation in order to maintain greater than 60% mortality. In the 8th generation, 7.5 PPM of Denim® produced 64.8% mortality, indicating that the insects had developed resistance to the insecticide. Resistance occurred rapidly in these selection studies but the overall resistance level in the 8th generation based on the change in our selection dose is only 7.5-fold.

LC_{50}s

LC_{50}s for two replicates of the susceptible strain and three replicates of the 8th generation selected strain are shown in Figure 3 (upper). For Rep 1 and Rep 2 of the parental (susceptible) strain, the LC_{50}s were 0.60 (0.21-1.28 95% confidence interval) and 0.99 (0.57-1.74) PPM, respectively. For the selected strain the LC_{50}s for Reps 1-3

were 6.37 (3.01-15.65), 6.6 (4.51-10.47) and 5.42 (1.36-31.35) PPM, respectively. The lower graph in Figure 3 is the combined results for all replicates where the LC_{50}s were 0.8 (0.46-1.34) and 6.1 (4.15-9.33) PPM for the parental and selected strains, respectively. This is a resistance ratio of 7.6-fold. It is clear from these results that selection in the laboratory with Denim by dipping results in the rapid development of larval resistance. The slope of the dose-mortality line for the selected strain suggests that greater resistance levels may be possible by additional selection. For example, 24% of the budworms survived the highest dose tested (12 PPM).

Feeding Disruption Assay for Denim Resistance in the Tobacco Budworm

This laboratory has been developing novel feeding disruption assays for monitoring resistance in Lepidoptera including the tobacco budworm (Bailey et al., 1998; Roe et al., 1999; Roe et al., 2000). The bioassay kit is now in the commercialization phase. The kit consists of a specially designed white plastic 16-well plate with recessed, hydrateable meal pads containing a diagnostic dose of insecticide and a blue indicator dye to monitor larval feeding (Figure 1). The appearance of blue feces easily seen on the background of the white plate is a measure of feeding rate. Neonates that produce blue feces (at diagnostic dose of insecticide) at a greater rate than a known susceptible population are diagnosed as being resistant. For fast acting insecticides like Denim, the end point can simply be mortality.

The relationship between the concentration of Denim in the meal pad hydration solution and fecal production by parental budworms is shown in Figure 4. As expected, as the Denim concentration increases, fecal production decreases. In the concentration range

of 0.0025 to 0.01 PPM, fecal production on the average decreased from 48 to 4 pellets per neonate, respectively. This concentration range was used as a diagnostic dose range to examine the use of the feeding disruption assay to monitor budworm resistance to Denim. A comparison of the number of fecal pellets produced on average by neonates of parental (susceptible) and selected budworms is shown in Figure 5. At all concentrations tested, the selected (resistant) budworms produced a greater number of fecal pellets than the parental strain as would be expected. The difference was greatest at the 0.0075 PPM dose; this dose appears to be an optimum dose for resistance monitoring using feeding disruption as a marker for resistance. However, Denim is fast acting and mortality was also a good end point for resistance detection. The advantage of the Feeding Disruption Assay plates in general, is that both endpoints can be used to measure resistance. Mortality at 0.0025, 0.005, 0.0075 PPM of Denim® was 16, 48, 54%, respectively, for the parental (susceptible) strain while no mortality occurred for the selected strain (Figure 6). Resistance was easily detected both by feeding disruption and mortality for Denim resistance levels of 7.6-fold, which demonstrates the sensitivity of this assay method for resistance detection.

Acknowledgments

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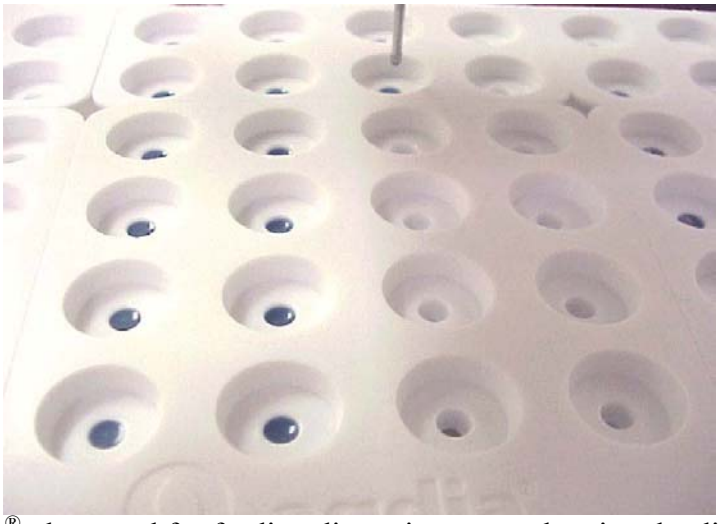

Figure 1: Agdia® plate used for feeding disruption assay showing the diet with blue indicator. (Used by permission from Norma Hoffman, Agdia® Inc.)

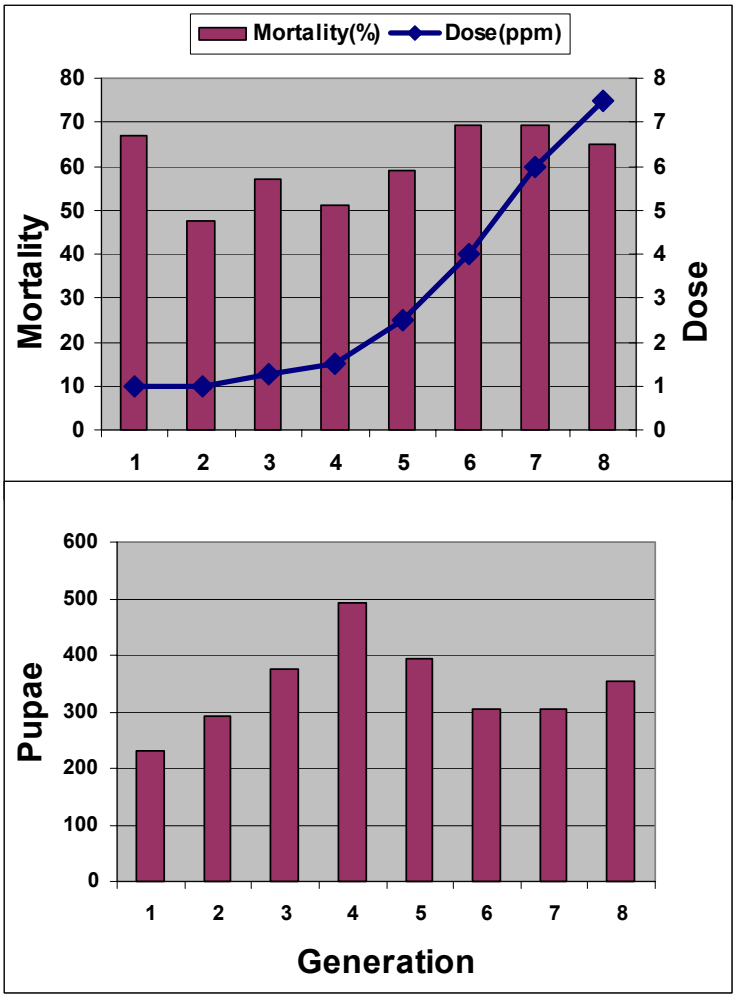

Figure 2. History of selection. Upper: Dose (PPM) and uncorrected mortality (%); lower: number of pupae per each generation of selection by larval dip using Denim®.

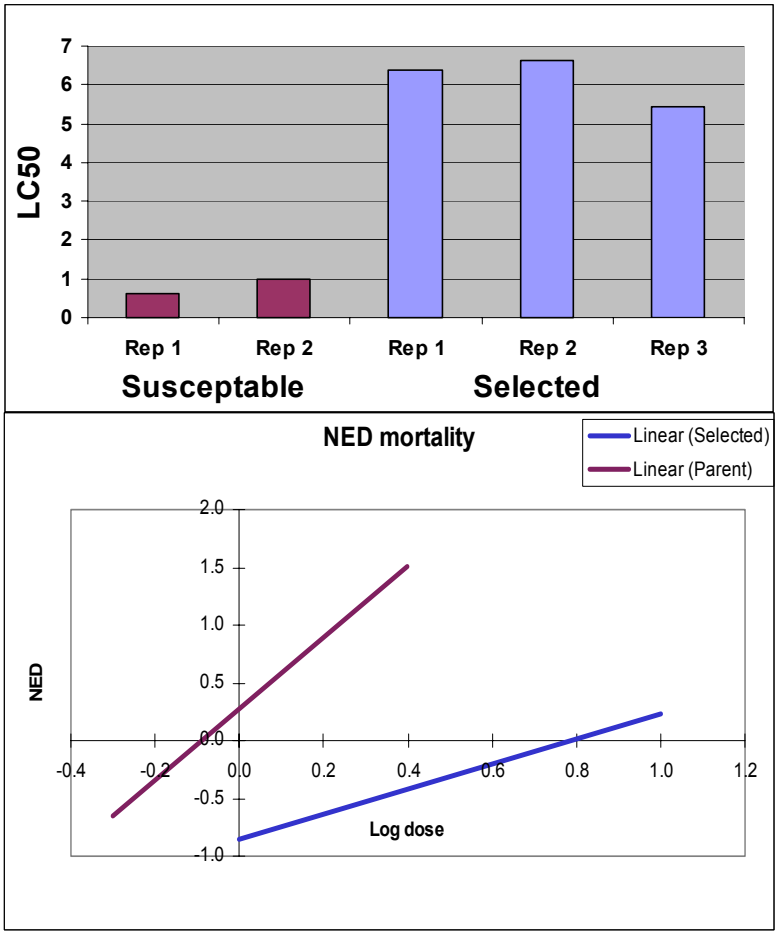

Figure 3. Upper: LC_{50}s for two replicates of the susceptible strain and three replicates of the 8th generation of the selected strain; lower: Log-dose probit plot of toxicity of Denim® using larval dip. NED (Noemal Equivalent Deviate) is probit minus 5.

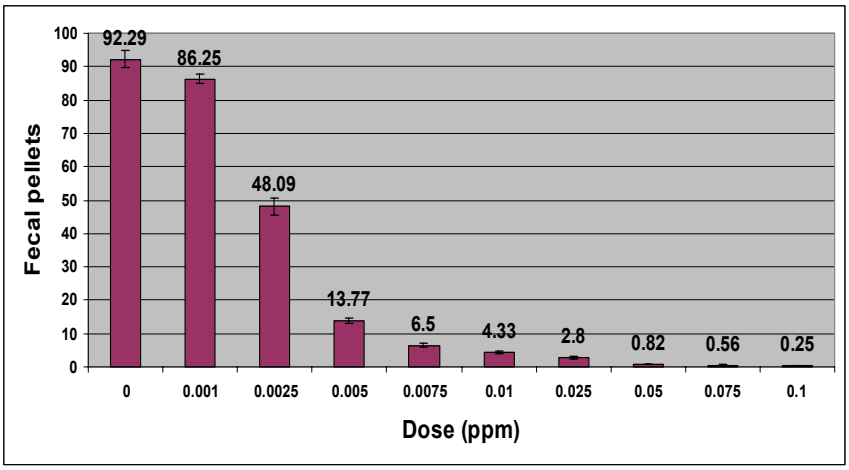

Figure 4. Feeding disruption assay using Agdia® plates showing the average number of fecal pellets produced per parental (susceptible) tobacco budworm neonate. Error bars represent ±1standard error of the mean.

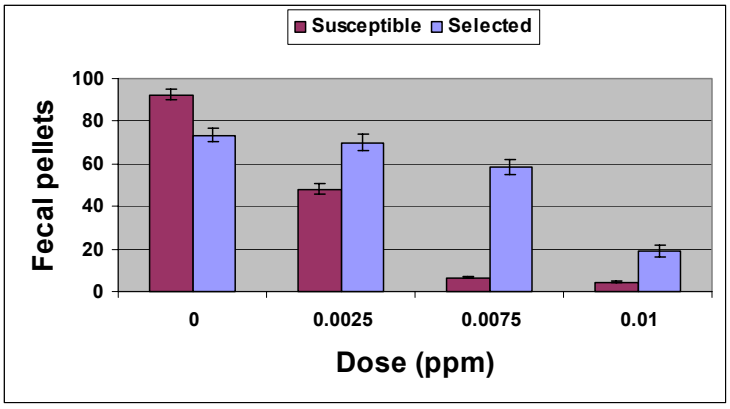

Figure 5. Comparison of number of fecal pellets produced per neonate for parental (susceptible) and selected tobacco budworms at different doses of Denim®. Error bars represent ±1 standard error of the mean.

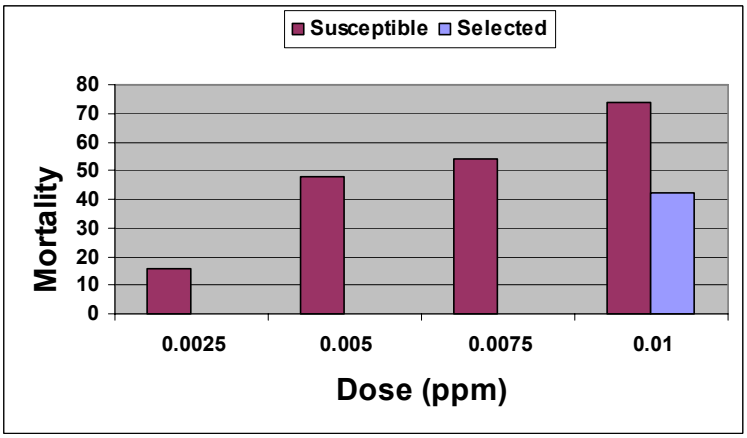

Figure 6. Neonate mortality for parental (susceptible) and selected budworms at different doses of Denim®. Thirty insects were used for each dose.