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Characterization of Hovi-mEH1, a microsomal epoxide hydrolase from the glassy-winged sharpshooter *Homalodisca vitripennis*

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Abstract

Epoxide hydrolase (EH) is an enzyme in the α/β -hydrolase fold superfamily that uses a water molecule to transform an epoxide to its corresponding diol. In insects, EHs metabolize among other things critical developmental hormones called juvenile hormones (JHs). EHs also play roles in the detoxification of toxic compounds that are found in the insect's diet or environment. In this study, a full-length cDNA encoding an epoxide hydrolase, Hovi-mEH1, was obtained from the xylem-feeding insect *Homalodisca vitripennis*. *H. vitripennis*, commonly known as the glassy-winged sharpshooter, is an economically important vector of plant pathogenic bacteria such as *Xylella fastidiosa*. Hovi-mEH1 hydrolyzed the general EH substrates *cis*-stilbene oxide and *trans*-diphenylpropene oxide with specific activities of 47.5 ± 6.2 and 1.3 ± 0.5 nmol of diol formed $\text{min}^{-1} \text{mg}^{-1}$, respectively. Hovi-mEH1 metabolized JH III with a V_{max} of 29.3 ± 1.6 nmol $\text{min}^{-1} \text{mg}^{-1}$, k_{cat} of 0.03 s^{-1} , and K_M of $13.8 \pm 2.0 \mu\text{M}$. These V_{max} and k_{cat} values are similar to those of known JH metabolizing EHs (JHEHs) from lepidopteran and coleopteran insects. Hovi-mEH1 showed 99.1% identity to one of three predicted EH-encoding sequences that were identified in the transcriptome of *H. vitripennis*. Of these three sequences only Hovi-mEH1 clustered with known JHEHs. On the basis of biochemical, phylogenetic, and transcriptome analyses, we hypothesize that Hovi-mEH1 is a biologically relevant JH-metabolizing enzyme in *H. vitripennis*.

Keywords

epoxide hydrolase; *Homalodisca vitripennis*; juvenile hormone; GWSS

Introduction

The leafhopper *Homalodisca vitripennis* (Hemiptera: Cicadellidae: Cixiinae), commonly called the glassy-winged sharpshooter (GWSS), is a polyphagous invasive species in California, Hawaii, and French Polynesia (Redak et al. 2004). GWSS feeds on the xylem tissues of plants to which it can efficiently transfer the bacterium *Xylella fastidiosa*. *X. fastidiosa* is a xylem-limited, plant pathogen that produces a biofilm that blocks the flow of water through the xylem tissues of wide range of economically important agricultural plants including grape and citrus (Hopkins and Purcell 2002). Two important plant diseases caused by *X. fastidiosa* are Pierce's Disease in grape and citrus variegated chlorosis in citrus.

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Epoxide hydrolase (EH) is a hydrolytic enzyme in the α/β -hydrolase fold superfamily that converts an epoxide to its corresponding diol (Morisseau and Hammock 2008). A microsomal EH (EC 3.3.2.9) known as JHEH metabolizes juvenile hormones (JHs) in insects. JHs are a group of epoxide containing sesquiterpenes that regulate critical biological events in insects including metamorphosis, reproduction, and behavior (Goodman and Granger 2005). EHs are also known to metabolize epoxide-containing xenobiotics and allelochemicals that are commonly found in the diet of polyphagous insects (Mullin 1988). Here we obtained an EH-encoding cDNA, *hovimeh1*, from a nymphal *H. vitripennis*. A recombinant EH, Hovi-mEH1, was expressed from this cDNA using a baculovirus expression vector and biochemically characterized. Based on its hydrolytic activity for JH, phylogenetic profile, and the transcriptome profile of *hovimeh1*, we hypothesize that Hovi-mEH1 may function as a biologically active JHEH in *H. vitripennis*.

Materials and Methods

Cloning of *hovimeh1* cDNA

The rearing conditions and source of the *H. vitripennis* that were used in this study are described previously (Rosa et al. 2012). Total RNA was isolated from the headless body (i.e., thorax and abdomen) of a single insect that was sacrificed at day 10 of the 5th instar using TRIzol Reagent (Invitrogen, Carlsbad, CA). One microgram of the total RNA was used to generate first strand cDNA and subsequently double-stranded cDNAs (20 amplification cycles) using a Creator SMART cDNA Library Construction kit (Clontech, Mountain View, CA). The double-stranded cDNAs were used as template for 3'- and 5'-RACE procedures (Kamita et al. 2013) to identify the 3'- and 5'-end sequences of *hovimeh1*. Primers JHEH7 (5'-CAYGGNTGGCCNGGNTCNGT-3') and GSWYrev (5'-CACCAATAACACTTGGATACCAACTGCCG-3') as well as reagents from the Creator SMART kit were used for 3'- and 5'-RACE, respectively. The JHEH7 primer was designed on the basis of a sequence of amino acid residues, HGWPGSV, that is highly conserved in known JHEHs.

Following the 3'- and 5'-RACE, a full-length, open reading frame that was predicted to encode an EH was amplified by PCR (Kamita et al. 2013) using the primers HoviEH5Bgl (5'-GAAGATCTATGATCAAGGGTGTGTTGGTGTC-3') and HoviEH3Eco (5'-CGGAATTCCTAGGTGGATTTGTGATGCAGAGC-3'). The HoviEH5Bgl and HoviEH3Eco primers placed *Bgl*II and *Eco*RI restriction endonuclease sites (underlined nucleotides) at the 5' and 3' ends, respectively, of the coding sequence cDNA. The 1.4 kbp-long amplicon was ligated into the pCR-Blunt vector (Invitrogen) generating pCR-Blunt-hovimeh1, and the sequence of the insert was determined in both directions. Subsequently, the 1.4 kbp-long insert was subcloned into the *Bgl*II and *Eco*RI sites of pAcUW21 (Weyer et al. 1990) generating the recombinant baculovirus transfer vector pAcUW21-hovimeh1.

Generation of recombinant AcHovimEH1 and protein expression

A recombinant baculovirus, AcHovimEH1, was generated following the transfection of Sf-9 cells (Invitrogen) with pAcUW21-hovimeh1 and *Bsu*36I-digested BacPAK6 baculovirus DNA (Clontech) using Cellfectin Transfection Reagent (Invitrogen). AcHovimEH1 was isolated from the supernatant of the transfected Sf-9 cells by three rounds of plaque purification on Sf-9 cells. The Sf-9 cells were cultured on ExCell 420 medium (SAFC Biosciences, Lenexa, KS) supplemented with 2.5% fetal bovine serum at 27°C. Recombinant HovimEH1 protein was produced in insect High Five cells (Invitrogen) that were grown in ESF921 medium (Expression Systems, Davis, CA). The High Five cells (1×10^6 cells ml⁻¹) were inoculated with AcHovimEH1 at a multiplicity of infection of 0.8 and collected by low speed centrifugation (230 $\times$ g) at 65 h post inoculation. Microsomes were

prepared as previously described (Kamita et al. 2013) from the AcHovimEH1-infected High Five cells and stored at -80°C . The relative amount of Hovi-mEH1 in the microsomal preparation was analyzed by image analysis of SDS-PAGE separated proteins as previously described (Kamita et al. 2013).

Enzyme assays for EH activity and determination of kinetic constants

Partition assays were used to determine the activity of Hovi-mEH1 for the general EH substrates *cis*-stilbene oxide (*c*-SO), *trans*-stilbene oxide (*t*-SO), and *trans*-diphenylpropene oxide (*t*-DPPO) (Morisseau and Hammock 2007); and for JH III (PerkinElmer, Boston, MA and Sigma-Aldrich, St. Louis, MO) (Hammock and Sparks 1977). Potential hydrolysis of the ester of JH III by contaminating esterases in the microsomal preparation was prevented by the addition of the esterase inhibitor OTFP into the JH III assay as previously described (Kamita et al. 2013). An LC/MS/MS protocol (Morisseau and Hammock 2007) was used to determine the specific activity of Hovi-mEH1 for the polyunsaturated fatty acid 14,15-epoxyeicosatrienoic acid (14,15-EET). The kinetic constants (Michaelis constant (K_M) and $V_{\max}$) of Hovi-mEH1 for JH III were determined using 8 substrate concentrations (0.642 to 69.8 μM) that were at least 5-fold below and 5-fold above the estimated K_M value. The assays were performed in triplicate and repeated at least three times for each concentration of substrate. Background hydrolytic activity was determined for each substrate and substrate concentration using microsomes prepared from control BacPAK6-infected High Five cells, and subtracted from the activity obtained with Hovi-mEH1 microsomes. The K_M and $V_{\max}$ were calculated using the Enzyme Kinetics module 1.1 of Sigma Plot (Systat Software, San Jose, CA). An estimated molecular weight of 52,108 Daltons was used to calculate the turnover value (k_{cat}).

Results and Discussion

Cloning and analysis of an EH-encoding cDNA from *H. vitripennis*

A full-length EH-encoding cDNA, *hovimeh1* (GenBank accession number JX855252), was identified by RACE and PCR procedures from a 5th instar *H. vitripennis*. *Hovimeh1* was 1,668 nts-long (Figure 1) and contained a 1,374 nts-long open reading frame flanked by 5'- and 3'-UTR sequences that were 174 and 101 nts-long, respectively. The deduced protein of *hovimeh1*, Hovi-mEH1 (Figure 1), was 458 amino acid residues and had a predicted mass of 52,108 Daltons and pI of 7.00. A membrane anchor domain of 23 amino acid residues was predicted at the N-terminal of Hovi-mEH1. The catalytic triad residues of Hovi-mEH1 were predicted to be D-227, H-432, and E-405. Other amino acid residues that are conserved in biologically active EHs including two tyrosine residues (Y-300 and Y-375) of the lid domain (oxyanion hole) and the HGWP motif (residues 152-155) were also found in Hovi-mEH1. Hovi-mEH1 showed 99.1% identity with "GWSS transcript 8298", one of three predicted EH encoding sequences that were found in the 5th instar nymphal transcriptome of *H. vitripennis* by next-generation sequencing (Nandety and Falk, 2013, unpublished). Of these three predicted EH encoding sequences, only Hovi-mEH1 (i.e., GWSS transcript 8298) clustered with JHEH and JHEH-related proteins from five insect orders (Figure 2). This phylogenetic analysis suggests that *H. vitripennis* encodes only one JH metabolizing EH.

Expression and purification of Hovi-mEH1

The recombinant baculovirus AcHovimEH1 produced at least 32 mg of Hovi-mEH1 per liter of High Five cells (2×10^6 cells ml^{-1}). Approximately, 91% of the total EH activity when measured using *c*-SO as a substrate was found in the microsomal fraction of AcHovimEH1-infected High Five cells. This microsomal localization indicated that the predicted membrane anchor domain of Hovi-mEH1 functioned to keep Hovi-mEH1 as a

transmembrane protein. Hovi-mEH1 represented approximately 11% of the total protein that was found in the microsomal preparation (Figure 3).

Hydrolytic activity of Hovi-mEH1 with general epoxide substrates

Hovi-mEH1 hydrolyzed the general EH substrate *c*-SO with a specific activity of 47.5 ± 6.2 nmol min⁻¹ mg⁻¹ (Table 1). In contrast, Hovi-mEH1 showed no detectable hydrolysis of the *trans* form of stilbene oxide (*t*-SO) under the same assay conditions; and more than 37-fold lower (in comparison to *c*-SO) activity for *t*-DPPO (Table 1). These findings suggested that Hovi-mEH1 has a preference for *cis*-epoxides. Hovi-mEH1 also showed 130-fold lower specific activity (in comparison to *c*-SO) for the polyunsaturated fatty acid 14,15-EET (Table 1).

Hydrolytic activity of Hovi-mEH1 with JH III

Hovi-mEH1 hydrolyzed JH III with a $V_{\max}$ of 29.3 ± 1.6 nmol of JH III diol formed min⁻¹ mg⁻¹ (Table 1). This $V_{\max}$ was similar to or slightly (2- to 3-fold) lower than that of authentic and recombinant JHEHs from lepidopteran insects such as *Manduca sexta* (i.e., MsJHEH (Touhara and Prestwich 1993)) and *Bombyx mori* (i.e., Bommo-JHEH (Zhang et al. 2005) or BmJHEH-r1 (Seino et al. 2010)) and the coleopteran *Tribolium castaneum* (TcJHEH-r3 (Tsubota et al. 2010)). The substrate turnover value (k_{cat}) of Hovi-mEH1 for JH III was 0.03 s⁻¹. This k_{cat} was similar or slightly faster than that of MsJHEH and Bommo-JHEH. The K_M value of Hovi-mEH1 for JH III was 13.8 ± 2.0 μM, a value that was 50-fold higher than that of MsJHEH (Touhara and Prestwich 1993) and 30-fold higher than that of Bommo-JHEH (Zhang et al. 2005). This high K_M resulted in a specificity constant (k_{cat}/K_M ratio) of Hovi-mEH1 for JH III of 2.2×10^3 M⁻¹ s⁻¹. In comparison, the k_{cat}/K_M ratios of MsJHEH and Bommo-JHEH are 130- and 30-fold higher, respectively, suggesting that JH III is a better substrate for the lepidopteran JHEHs. Although JH III is the most common form of JH that has been identified in insects to date, it may not be the endogenous JH in hemipteran insects. A novel JH, JH III skipped bisepoxide JH, was reported from the hemipteran *Rhodnius prolixus* (Kotaki et al. 2009). This or another form of JH that is endogenous to *H. vitripennis* may generate a higher k_{cat}/K_M ratio with Hovi-mEH1 as was found for MsJHEH (Touhara and Prestwich 1993). Hovi-mEH1 may also be a useful tool to further characterize novel JHs such as JH III skipped bisepoxide JH from hemipteran insects.

In summary, we have identified for the first time a microsomal EH, Hovi-mEH1, from the economically significant hemipteran *H. vitripennis*. On the basis of its high velocity for JH, phylogenetic profile, and the transcriptome profile of *hovimeh1*, we believe that Hovi-mEH1 is the biologically active JHEH of *H. vitripennis*.

Acknowledgments

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Abbreviations

14,15-EET	14,15-epoxyeicosatrienoic acid
AcHovimEH1	recombinant <i>Autographa californica</i> baculovirus expressing Hovi-mEH1
Bommo-JHEH and BmJHEH-r1	JHEH from <i>Bombyx mori</i>
c-SO	cis-stilbene oxide
EH	epoxide hydrolase
Hovi-mEH1	microsomal EH from <i>H. vitripennis</i>
hovimeh1	full-length EH-encoding cDNA from <i>Homalodisca vitripennis</i>

JH	juvenile hormone
JHEH	juvenile hormone epoxide hydrolase
MsJHEH	JHEH from <i>Manduca sexta</i>
OTFP	3-octylthio-1,1,1-trifluoropropan-2-one
<i>t</i>-DPPO	<i>trans</i> -diphenylpropene oxide
<i>t</i>-SO	<i>trans</i> -stilbene oxide

cattatattcagtgagtcgaagctgctttagttacgagtaagactgagacag
 ataagtcacatcccaactctgttctgttctggcggaagttttgtgaatacattctctt
 atcctagctcaaacctgttcttaccctgtgtccaataaatttttttagctattgccaca
 atgatacaaggtgtgtgtgtgtcagtgctagtggtgtgtgtcagttgcatggggcta
 M I K G V L V S V L V V V S A V A L G L 20
 tacatagactacaacaatgctttcactggtacagccccatcaccaataatgcaatggtgg
 Y I D Y N N A F T G T A P S P I M Q W W 40
 ggagaaggttaaacctagattagatgatggcatcatcaaacattcaaaatcaactttccc
 G E G K P R L D D G I I K P F K I N F P 60
 aaagagcttctggatgatttgcatcatcgtctcagcaacactcgccctttccaccacca
 K E L L D D L H H R L S N T R P F H P P 80
 ctacagcagtggtgttccagtatggatttaacacagagtttctgcagactgtcctggac
 L D D V G F Q Y G F N T E F L Q T V L D 100
 ttttgagaacgaaatactcctgggaggaacgtcaaaattacttgaactccctgcctcaa
 F W R T K Y S W E E R Q N Y L N S L P Q 120
 tacacaatggaaactcaagcctgcagatccacttcacatgtaaaacaaaaatgca
 Y T M E T Q G L Q I H F I H V K P K N A 140
 aaaggattacatgtatatccactcctgctagtcctggttgccaggttctgtgagagaa
 K G L H V Y P L L L V **H G W P** G S V R E 160
 ttttatgatcatccctctgctgactactccaagagaaggacaagattttgtgtttgag
 F Y D I I P L L T T P R E G Q D F V F E 180
 gtattgtcccatcactgcctggctatggattttcacaggctacaacccgacatggtctt
 V V A P S L P G Y G F S Q A T T R H G L 200
 ggaccagccaggtcgctgttcttgcgactcttatgaagcaactgggtcacaaaagg
 G P A Q V A V V L R T L M K Q L G H K R 220
 tactatgtccagggcggagattggggcagcatcattgtcactctgcttgacagatgacc
 Y Y V Q G G **D** W G S I I V T L L A Q M T 240
 cgtggtgatagattcttggacttcacacaaatagcgctatgttatgccaactactgga
 R G D R I L G L H T N M A Y V M P T T G 260
 atgatggcgtggcaaatcatcgccagttggtatccaagtgttattggtgatgaacagaa
 M M A W Q I I G S W Y P S V I G D E T E 280
 cagaagtatgtttatccaatgtctgatcactactcctttttagtagaggaatctggatat
 Q K Y V Y P M S D H Y S F L L E E S G **Y** 300
 ttgcactctgcaagctaccaagcctgcacaaataggtgtagctttgagagattcacccgatg
 L H L Q A T K P D T I G V A L R D S P M 320
 gggctggctgtacatcttgagaagtctcacttggaactgatcggttcaggaagagt
 G L A A Y I L E K F S T W T D R S W K S 340
 ctcaagatggaggtcttcttaaaagtccaagctggaagctctattggacaatgtcatg
 L K D G G L L K K F K L E A L L D N V M 360
 atctactggtgacagccacatcactacctccatgcgtctgtacagtgaacactcaac
 I Y W M T G T I T T S M R L **Y** S E H F N 380
 aggccccatcgagcattgggctttgaccaggcaccagtaaaagtacctacggcgatagct
 R T H R A L G F D Q A P V K V P T A I A 400
 cgtttcccaatgagctcgcaatcagcctcgatgcttttagagaaaaagttcacgaat
 R F P N **E** L A N Q P R C F L E K K F T N 420
 ttggtgcaagtaacagacatgcctacagaggtcactttgcagcctttgaggaacctaaa
 L V Q V T D M P T G G **H** F A A F E E P K 440
 ctgttggctgatgatatttggattttgtcagcgtctgcacacaaatccacctaggcc
 L L A D D I W D F V S A L H H K S T * 458
 acctgcccaagcctacacaaagtgtacaacaataattataatcaaaagactgactttta
 gtatacatttcttaccataaagccttattactttgttaaaaaaaaaaaaaaa

Fig. 1.

Nucleotide (lower case text) and deduced amino acid (upper case text) sequences of *hovi-meh1* and Hovi-mEH1, respectively. The 5'- and 3'-UTR sequences, and coding sequence of *hovi-meh1* were 174, 101, and 1,374 nts-long, respectively. Amino acid residues that form the putative catalytic triad (D-227, H-432, and E-405), oxyanion hole (Y-300 and Y-375), and HGWP motif (residues 152-155) are shown in bold text. The asterisk indicates a stop codon (TAG). A putative membrane anchor domain (residues 2-24) that was predicted by SOSUI version 1.11 (Hirokawa et al. 1998) is shown in italic text. A predicted cleavage and polyadenylation specificity factor (CPSF) complex binding site is underlined. Amino acid residue positions are indicated to the right.

Fig. 2. Phylogenetic analysis of EH sequences from *H. vitripennis* and five other insect orders. The GenBank accession number of each protein sequence is given within the parentheses. The phylogenetic analysis was performed using MEGA version 5.05 (Tamura et al. 2011) under the parameters described previously (Kamita et al. 2013).

Fig. 3.

Analysis of the purity of Hovi-mEH1 microsomal preparations by SDS-PAGE. In this representative example 2.4, 4.8, and 9.6 μ g of the microsomal preparation from AcHovimEH1-infected High Five cells (lanes 1, 2, and 3, respectively) were separated by SDS-PAGE (10% NuPAGE Bis-Tris gel) and stained. The arrowhead indicates the migration of Hovi-mEH1 (52.1 kDa). In this example, Hovi-mEH1 was estimated to represent 11% of the total protein by image analysis using ImageJ software (W. S. Rasband, 2006, U. S. National Institutes of Health, <http://rsb.info.nih.gov/ij/>). The lanes marked M show the migration of protein standards (SeeBlue Plus2, Invitrogen); their estimated sizes are indicated between the panels in kDa. Lane 4 shows the migration of a microsomal preparation (17.7 μ g) from control BacPAK6-infected High Five cells.

Table 1
Hydrolytic activity of Hovi-mEH1 for various epoxide-containing substrates

Substrate	Structure	Activity ^a (nmol min ⁻¹ mg ⁻¹)
<i>c</i> -SO		47.5 ± 6.2
<i>t</i> -SO		n.d. ^b
<i>t</i> -DPPO		1.3 ± 0.5
14,15-EET		0.36 ± 0.007
JH III ^c		29.3 ± 1.6

^aThe enzyme assays were performed at 30°C in buffer (100 mM sodium phosphate, pH 8.0, for JH III, *c*-SO, *t*-SO, and *t*-DPPO; or 25 mM Bis-Tris, pH 7.0, for 14,15-EET) containing substrate (50 μM for *c*-SO, *t*-SO, *t*-DPPO, and 14,15-EET; or 0.642 to 69.8 μM for JH III), 1% (v:v) ethanol, and 0.1 mg ml⁻¹ BSA. The JH III assays also contained 10 μM OTFP and 2% (v:v) ethanol. The values shown are the mean ± standard deviation of at least three separate experiments. Background hydrolytic activity in negative control experiments was less than 1% of total activity.

^b Activity was not detected at an assay detection limit of 0.01 nmol min⁻¹ mg⁻¹.

^c V_{max} is shown for JH III.