Expression and characterization of the recombinant juvenile hormone epoxide hydrolase (JHEH) from *Manduca sexta*

Stéphane Debernard [a,b], Christophe Morisseau [a], Tonya F. Severson [a], Li Feng [c], Hubert Wojtasek [c,d], Glenn D. Prestwich [c], Bruce D. Hammock [a,*]

[a] *Departments of Entomology and Environmental Toxicology, University of California, Davis, CA 95616, USA*
[b] *Present Address: Laboratoire de Physiologie Cellulaire des Invertébrés, Université Pierre et Marie Curie, 12 rue Cuvier 75005, Paris, France*
[c] *Department of Medicinal Chemistry, University of Utah, Salt Lake City, UT 84112, USA*
[d] *Present Address: National Institute of Sericultural and Entomological Science, 1-2 Ohwashi, Tsukuba, Ibaraki 305, Japan*

Abstract

The cDNA of the microsomal Juvenile Hormone Epoxide Hydrolase (JHEH) from *Manduca sexta* was expressed in vitro in the baculovirus system. In insect cell culture, the recombinant enzyme (Ms-JHEH) was produced at a high level (100 fold over background EH catalytic activity). As expected, Ms-JHEH was localized in the microsomal fraction with a molecular mass of approximately 50 kDa. Ms-JHEH showed a substrate and inhibitor spectrum similar to the wild type JHEH isolated from eggs of *M. sexta*. Its enzymatic activity was the highest for Juvenile Hormone III. Ms-JHEH hydrolyzed several *trans*-epoxides faster than *cis*-epoxides. A putative hydroxyl-acyl enzyme intermediate was isolated suggesting a catalytic mechanism of Ms-JHEH similar to the mammalian EHs. © 1998 Elsevier Science Ltd. All rights reserved

1. Introduction

Growth and development of larval insects as well as the reproductive function of adult insects are regulated, in part, by Juvenile Hormone (JH; see Fig. 1). The JH concentration in insects is regulated by two dynamic processes, biosynthesis and degradation (Hammock, 1985; Roe and Venkatesh, 1990). The major routes of JH metabolism in Lepidoptera are ester hydrolysis by JH esterase (JHE; EC 3.1.1.1) and epoxide hydration by JH epoxide hydrolase (JHEH; EC 3.3.2.3). These catabolic pathways generate three metabolites (JH-acid, JH-diol and JH-acid-diol) that are each believed to be physiologically inactive (Hammock, 1985). Most of the research on JH metabolism has concentrated on the mechanism of action of JHE. In comparison, little is known about insect JHEH. However, JHEH seems to have a critical role in insect development. For example,

Lassiter et al. (1995) have found a dynamic role for JHEH to the exclusion of JH esterase in metamorphosis of *Culex quinquefasciatus* while both JHE and JHEH appear important in cyclorrhaphous flies (Campbell et al., 1992). In several stages of larval *Manduca sexta*, the formation of diol is the major in vivo route of JH metabolism (Halarnkar et al., 1993), which is followed by the conversion of the diol to phosphate conjugates (Grieneisen et al., 1995).

EH activity in insects has been partially characterized in *Drosophila melanogaster* (Harshman et al., 1991), *M. sexta* (Casas et al., 1991), *Galleria mellonella* (Wisniewski et al., 1986) and *Trichoplusia ni* (Wing et al., 1981; Roe et al., 1996). The first insect EH was purified from the larval midgut of *Spodoptera eridania* (Mullin and Wilkinson, 1980a). The enzyme was very active on monosubstituted epoxides, however, activity on JHs was not demonstrated (Mullin and Wilkinson, 1980b). One of the authors suggested the presence of different groups of EH's in insects based on substrate selectivity (Mullin, 1988). A microsomal EH was isolated from eggs of *M. sexta* which effectively hydrolyzed
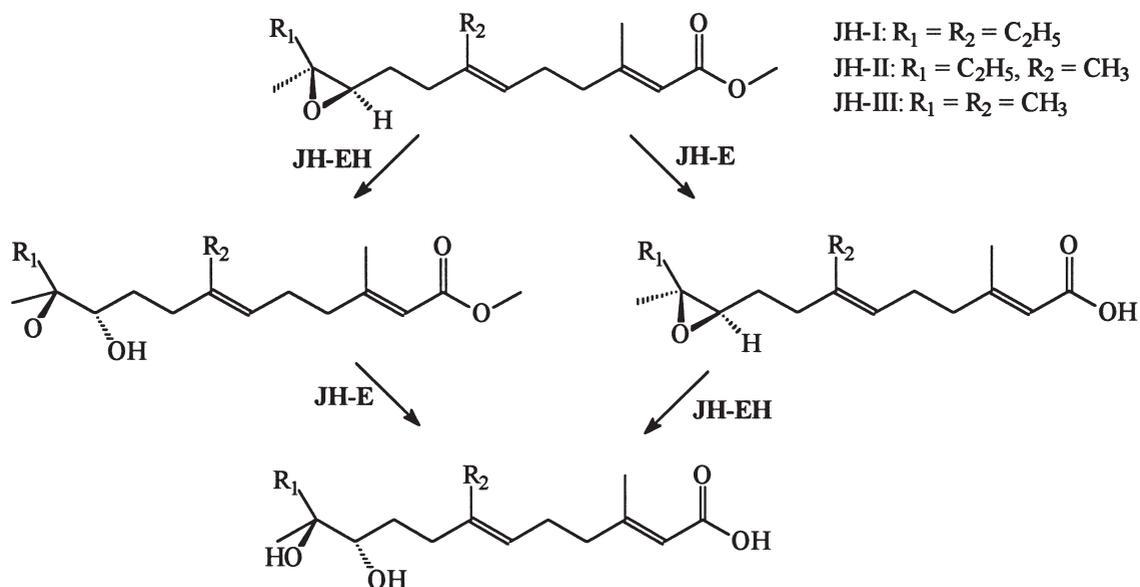

Fig. 1.   Hydrolysis of juvenile hormone by JH epoxide hydrolase (JHEH) and JH esterase (JHE).

JHs and was specifically labeled by JH photoaffinity analogs (Touhara and Prestwich, 1993). The same group recently reported the cloning of the cDNA of this protein in *E. coli* (Wojtasek and Prestwich, 1996). However, no active enzyme has been produced using this clone. The authors reported an amino-acid sequence with 44% identity and 64% similarity to human microsomal epoxide hydrolase. Unlike insect EHs, mammalian EHs are well known (for review: Hammock et al., 1997). They are α,β hydrolase fold enzymes (Ollis et al., 1992) with a two-step mechanism involving the formation of a covalent acyl-enzyme intermediate (Lacourciere and Armstrong, 1993; Hammock et al., 1994; Borhan et al., 1995a). Herein, we report the expression of active JHEH from *M. sexta* in the baculovirus expression system, the characterization of its substrate spectrum and the determination of its mechanism.

2. Materials and methods

2.1. Chemicals and materials

[^{14}C]-*cis*-9-10-Epoxystearic acid (ESA), [^{3}H]-*trans*-1,3-diphenylpropene oxide (t-DPPO) and [^{3}H]-*cis*-1,3-diphenylpropene oxide (c-DPPO) were previously synthesized in this laboratory (Borhan et al., 1994, 1995b). [^{3}H]-Juvenile hormone III (JH-III) was purchased from Amersham Life Science (Arlington Heights, IL). [^{3}H]-*Trans*-stilbene oxide (t-SO) and [^{3}H]-*cis*-stilbene oxide (c-SO) were synthesized in this laboratory (Gill et al., 1983). Unlabeled t-DPPO was synthesized as described by Borhan et al. (1994). [^{3}H]- Epoxybishomofarnesyl diazoacetate (EBDA) was synthesized by Dr. I. Ujvári

and Dr. G. Dorman at the University Stony Brook, NY (Ujvári et al., 1990). [^{3}H]-Epoxyhomofarnesyl diazoacetate (EFDA) was previously synthesized (Prestwich et al., 1984). Other chemicals were purchased from Aldrich Chemicals (Milwaukee, WI) and used without any further purification. Liquid scintillation cocktail (CytoScint) was purchased from Fisher Scientific (Fairlawn, NJ). Bicinchoninic acid (BCA) reagent for protein concentration determination was obtained from Pierce, Inc. (Rockford, IL).

2.2. Polymerase chain reaction (PCR) and PCR product cloning

Polymerase chain reactions were run on a PTC-100 programmable thermal cycler (MJ Research) using recombinant *Pfu* DNA polymerase (Stratagene) which creates blunt-ended fragments. Reactions were carried out in 100 μl final volume including 10 mM KCl, 6 mM ammonium sulfate, 20 mM Tris-HCl (pH 8), 2.5 mM MgCl$_2$. Each primer was added thereafter at 1 μM, each dNTP at 0.8 mM and 1 ng DNA template with 2.5 units of *Pfu* polymerase. Following an initial denaturation of 95°C for 5 min, the thermal amplification procedure consisted of 25 cycles of denaturation 1 min at 95°C, annealing at 60°C for 30 s (high stringency PCR conditions) and extension at 72°C. The extension time varied from 30 s to 1 min according to the size of the fragment to be amplified. The blunt-ended PCR product was purified by agarose gel electrophoresis and ligated into pCR-Script™ SK(+) amp vector using Stratagene's pCR-Script™ SK(+) cloning kit following the manufacturer's instructions and then transformed into competent cells of *Escherichia coli*. Following colony isolation,

DNA minipreps were prepared and correct insertion was determined by restriction enzyme analysis. The DNA from the clone containing the insert was sequenced to verify amplification of the appropriate coding sequence. The sequencing was performed by the "dideoxy chain termination" method (Sanger et al., 1977) using Sequenase II (United States Biochemical Corp).

2.3. Construction of the recombinant virus

A pair of DNA primers (A1, A2) were synthesized for use in the PCR to amplify the ORF of Ms-JHEH cDNA from the plasmid pBEH, constructed by Wojtasek and Prestwich (1996):

A1:5′-primer (5′-*AGATCT*GATGTACAAAATAC-TATCGTCATTC, bp 145–169 of pBEH)

A2:3′-primer (5′-*GAATT*CATCAGCTGGATTTTT-GGTTTCTCTTTC, bp 1509–1535 of pBEH).

Each primer contained a 5′-*Eco*R I or *Bgl* II restriction enzyme site (italicized) and 25 or 27-base specific region to the Ms-JHEH cDNA sequence. The 1403-bp PCR product obtained (P1) was purified and ligated into a pCR-Script vector. The correct clone pS1EH was sequenced and was found to be identical to the ORF of Ms-JHEH cDNA sequence except for a 5-bp change that was most likely due to polymerase misincorporation. The mutations were localized at the 3′-end of ORF in a region designated R1 delimited by Aat II cleavage at the position bp 1247 of pBEH and concurrent cleavage by *Eco*R I. A new 5′-primer designed:

A3: 5′-*GACGTC*AACGCGCCTGTACGCCGAGGG, bp 1243–1269 of pBEH;

containing a 5′-*Aat* II restriction enzyme site with 3′-primer (A2) allowed the amplification of a 299-bp PCR product (P2) from pBEH. P2 was cloned into pCR-Script and the sequencing of resulting pS2EH clone did not show any mutations in the R1 region. P2 was excised from pS2EH by digestion with *Aat* II and *Eco*R I, purified on low melting temperature agarose to be cloned into pS1EH to replace the mutated R1 region. The isolated pS3EH clone contained the entire ORF of Ms-JHEH cDNA without any mutation. The plasmid pAcUW21 was opened by double digestion with *Bgl* II and subsequently *Eco*R I. The insert of pS3EH was excised by digestion with *Bgl* II and *Eco*R I, purified and ligated into the *Bgl* II and *Eco*R I sites of plasmid pAcUW21 under the control of a P10 promotor, forming the baculovirus cotransfecting plasmid pAcUW21-mEH. For the transfection, plasmid pAcUW21-mEH was purified by centrifugation on a cesium chloride, ethidium bromide equilibrium gradient (Sambrook et al., 1989). To obtain the recombinant virus, SF-21 cells (10⁶ cells/ml) were co-transfected in a 35 mm tissue culture dish with 0.5 μg of pAcUW21-mEH and 0.5 μg of Bac-PAK6 viral DNA linearized according to standard protocol (O'Reilly et al., 1992). Cell transfections were mediated by lipofectin (Life Technologies). The putative recombinant viruses were purified by plaque purification as described (O'Reilly et al., 1992). Recombinant virus (SDCM) were selected by EH activity produced in SF-21 cells infected with recombinant virus.

The rat microsomal epoxide hydrolase (RmEH) virus was prepared in an identical way to the Ms-JHEH virus using the cDNA for the microsomal epoxide hydrolase from Wistar rats provided by Franz Oesch and Michael Arand (Mainz, Germany; unpublished data). This was placed under the P10 promoter using pAcUW21 (unpublished data), yielding a virus similar but not identical to that described by Lacouciere et al. (1993). The Lac Z virus was prepared identically (Grant et al., 1996).

2.4. Cell culture and production of the epoxide hydrolase

Cells were routinely grown at 27°C, in 100 ml of complete medium [*T. ni* H-5: 99% EX-CELL 405 with L-glutamine (JRH Biosciences, Lenexa, KS) and 1% Pen/Strep antibiotics (Sigma). SF-21: EX-CELL 401 with L-glutamine; 3% heat-inactivated fetal bovine serum (Intergen, Purchase, NY) and 1% Pen/Strep antibiotics] in a 250 ml Ehrlenmeyer or spinner flask respectively. A culture was seeded with a cell density of 1.5×10^5 cells/ml until it reached 3×10^6 cells/ml. Cells were used for baculovirus expression while in the exponential phase of growth, usually 1×10^6 cells/ml. For enzyme production, cells (5×10^5 cells/ml) were infected with viral solution at a multiplicity of infection of 0.1. Lac Z and RmEH viruses were used as negative and positive control, respectively, of EH activity production. The control viruses were also infected with a multiplicity of infection of 0.1. Every day, during five days, 5 ml samples were taken from the culture. Cells were harvested by centrifugation (100 g for 10 min). The pellet was resuspended in 3 ml of Tris/HCl buffer 50 mM pH 8.0 containing 1 mM of PMSF, EDTA and DTT (buffer A). The cells were disrupted using a Polytron homogenizer (9000 rpm for 30 sec). The homogenate was centrifuged at 4000 g for 10 min. EH activity (JH-III for Lac Z and Ms-JHEH, and c-SO for RmEH) and protein concentration of the supernatant were measured.

2.5. Sub-cellular localization

After five days of incubation, *T. ni*-H5 cells were harvested from 200 ml of medium as indicated above. They were suspended in 20 ml of buffer A and disrupted using a Polytron homogenizer (9000 rpm for 2 min). The homogenate was centrifuged at 1000 g for 15 min at 4°C, and the pellet was resuspended in buffer A (10 ml) and spun at 1000 g during 15 min. The mixture of supernatants (crude extract) was centrifuged at 12 000 g for

20 min. The pellet was washed twice by resuspending in 5 ml buffer and centrifuged at $12\,000\,g$ for 20 min. The final pellet (mitochondrial/peroxisomal fraction) was resuspended in buffer A. The $12\,000\,g$ supernatants were centrifuged at $100\,000\,g$ for 1 h at $4°C$. The pellet was washed by resuspending in buffer A and centrifuged at $100\,000\,g$ for 30 min. The resulting pellet (microsomal fraction) was resuspended in 5 ml of buffer A containing 0.2% of Triton x 100. The protein concentration was estimated by BCA assay using BSA as a standard.

2.6. Enzyme assays

Epoxide hydrolase activity of the cell extracts was measured using [^{3}H]-JH-III as described previously (Mumby and Hammock, 1979a). Briefly, 100 μl of cell extracts diluted in Tris/HCl buffer (50 mM pH = 8.0) containing 0.1 mg/ml of BSA were incubated at $30°C$ for 2 min. 1μl of 5mM JH-III solution in ethanol was added (final concentration: 50 μM). The mixture was incubated at $30°C$ for 10 min. The reaction was quenched by addition of 100 μl of methanol. The remaining epoxide was extracted by 250 μl of iso-octane. The quantity of diol formed was determined using a liquid scintillation counter (Wallac Model 1409; Gaithersburg, MD) to quantify the radioactivity contained in the water phase. This value was corrected for the amount of epoxide partitioning into the aqueous phase. One unit of epoxide hydrolase corresponds to the amount of enzyme which catalyzed the formation of one μmol of diol per minute in the above conditions.

EH activity of microsomal fraction was also measured using [^{3}H]-labeled *trans*-1,3-diphenylpropene oxide (t-DPPO), *cis*-1,3-diphenylpropene oxide (c-DPPO), *trans*-stilbene oxide (t-SO), *cis*-stilbene oxide (c-SO) and [^{14}C]-labeled *cis*-9,10-epoxystearic acid (ESA) as described previously (Wixtrom and Hammock, 1985; Borhan et al., 1995b). EH activity was measured on JH-I and JH-II as described by Touhara and Prestwich (1993).

2.7. Polyacrylamide gel

SDS/PAGE was conducted on 1 mm thick slab gels consisting of a 10% acrylamide resolving gel at pH = 8.8 and a 4% acrylamide stacking gel at pH = 6.8 in the presence of 0.1% of sodium dodecyl sulfate (Laemmli, 1970). The samples were heated with 1% SDS and 1% β-mercaptoethanol at $100°C$ for 2 minutes. The reference proteins were: phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa. Proteins were stained with 0.1% Coomassie Brillant Blue R-250.

2.8. Photo-affinity labeling and fluorography

Photo-affinity labeling was performed as described previously (Prestwich et al., 1987). Briefly, the protein samples were labeled by [^{3}H]-epoxybishomofarnesyl diazoacetate (EBDA) and [^{3}H]-epoxyhomofarnesyl diazoacetate (EFDA) using polyethylene glycol 20 000-coated quartz tubes. The labeled proteins were separated on an SDS-polyacrylamide gel as described above. The fluorography was carried out as described previously (Mohamed et al., 1989).

2.9. Labeling of the enzyme

Enzyme labeling was performed as described previously (Pinot et al., 1995). Briefly, 1 μl of radioactive substrate ([^{3}H]-cDPPO: final concentration: 34.5 μM; 100 cpm/pmol) was added to 100 μl of enzyme solution in the indicated buffer containing 0.1 mg.ml^{-1} of BSA in a 6×50 mm glass tube held in an ice bath. The solution was mixed for 3 s at room temperature and the reaction was stopped by precipitating the protein with trichloroacetic acid (20% final concentration). The proteins were harvested by centrifugation ($10\,000\,g$ for 20 min at $4°C$). The pellet was washed two times with 200 μl cold acetone. The final pellet was dissolved in 10% (w/v) SDS (50 μl), and 1.2 ml of scintillation liquid were added. The radioactivity incorporated into the protein was determined in a Liquid Scintillation Counter.

3. Results

3.1. Construction of the Ms-JHEH recombinant virus

The cDNA sequence of Ms-JHEH from *Manduca sexta* consists of 1833 bp containing 145 bp of untranslated 5′-region, 1386 bp of the ORF coding for 462 amino acids and 302 bp untranslated 3′-fragment (Wojtasek and Prestwich, 1996). The untranslated 5′-region is long (over 30 nucleotides) and contains additional ATGs that are not intended for initiation purposes. These factors could interfere in the level of expression of the heterologous gene in the baculovirus expression system (O'Reilly et al., 1992). A polyadenylation signal is already present in the transfer plasmid pAcUW21 and did not need to be supplied with untranslated 3′-region of heterologous gene. The addition of another polyadenylation signal might affect the efficiency of RNA processing and/or RNA stability (O'Reilly et al., 1992). Also, in order to optimize the expression, the two non-coding sequences were eliminated and only the ORF of Ms-JHEH cDNA was inserted into pAcUW21. This strategy was performed by PCR using the *Pfu* DNA polymerase because of the small size of the ORF to amplify and the high fidelity amplification

of *Pfu* DNA polymerase reducing the risk of mistakes. The ORF was amplified by PCR using primers (A1,A2) containing a 5′-*Eco*R I or *Bgl* II restriction site to be cloned into *Bgl* II and *Eco*R I cloning sites of pAcUW21 under the control of the P10 promoter. The blunt-ended PCR product (P1) obtained was cloned into pCR-Script vector and the correct clone pS1EH was sequenced (Fig. 2A). The subcloning into pCR-Script vector was an additional step to verify the sequence of PCR fragment and to generate *Eco*R I, *Bgl* II cohesive ends through excising the fragment by digestion with *Bgl* II and *Eco*R I.

The sequence of pS1EH revealed the incorporation of mistakes localized at the 3′ end of ORF in region named R1 delimited by Aat II at the position bp 1247 of pBEH and *Eco*R I (Fig. 2A). All the catalytic residues implicated in the mechanism of epoxide hydration in mammalian and plant EHs are conserved in Ms-JHEH. In the enzyme from *Manduca sexta*, this catalytic triad could correspond to Asp^{227}, His^{428} and Asp^{350}/Glu^{401} (Wojtasek and Prestwich, 1996). The mutations in the R1 region modified the C-terminal portion of protein with the disappearance of amino acids His^{428} and Glu^{401}. To conserve the activity of Ms-JHEH, the region R1 was replaced by the PCR product P2 obtained by amplification from pBEH with primers (A2, A3) (Fig. 2B). The resulting clone pS3EH contained the ORF of Ms-JHEH without any mistake (Fig. 2C), and the insert was cloned into pAcUW21 to form the baculovirus cotransfecting plasmid pAcUW21-mEH (Fig. 2D). After the cotransfection of SF-21 cells with pAcUW21-mEH and Bac PAK6 viral DNA, 66% of the purified recombinant viruses demonstrated EH activity which was varying in activity over a ten fold range (data not shown). The recombinant virus SDCM1 with the highest activity was selected.

3.2. Expression and localization

The expression of the recombinant Ms-JHEH was followed at time-points during the growth of SF-21 and *T.ni*-H5 cells infected with the recombinant virus (Fig.
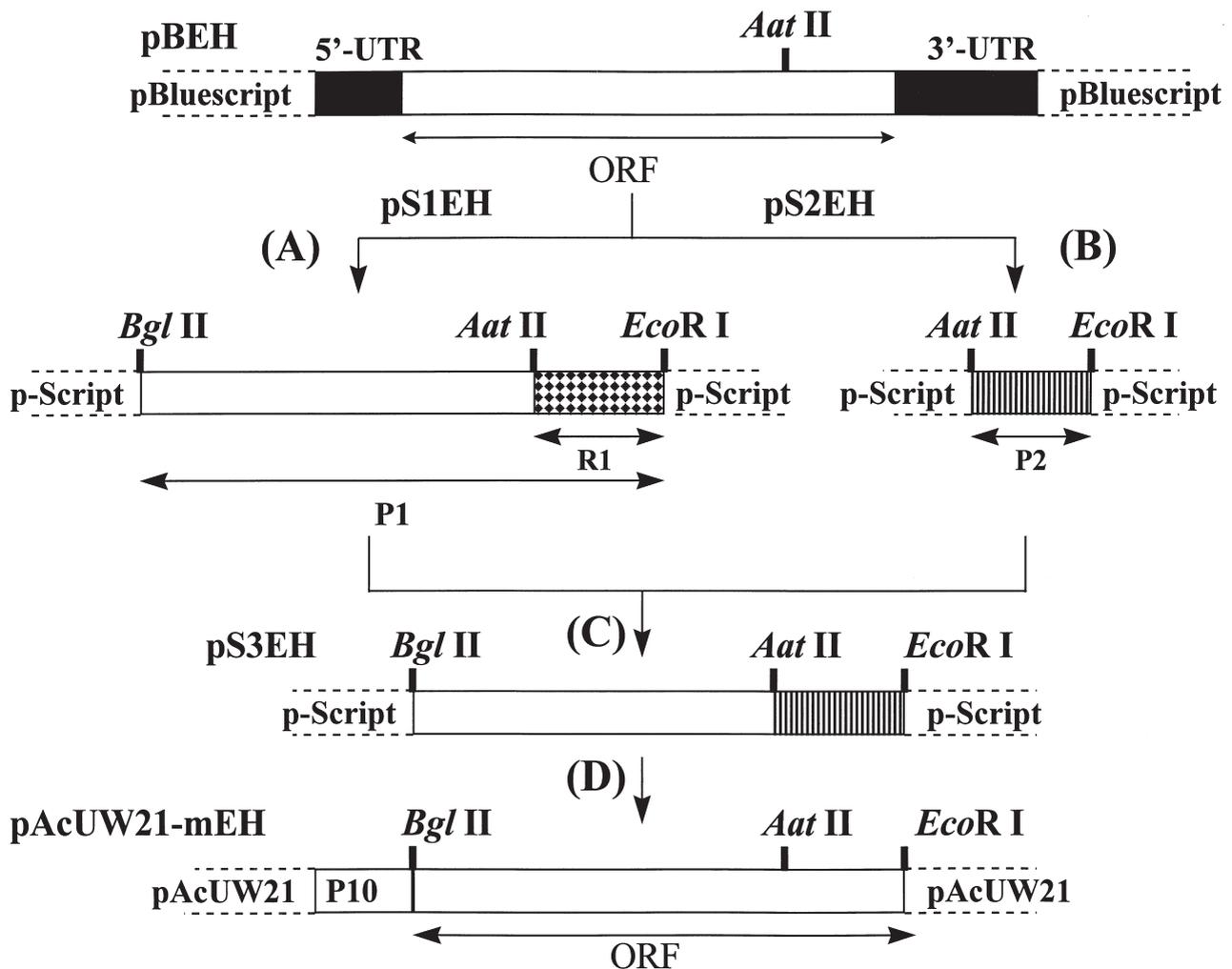

Fig. 2.　Construction of recombinant transfer vector pAcUW21-mEH.

3). Epoxide hydrolase activity appeared after the second day of incubation for both cell lines, and reached a plateau of production after four days (Fig. 3A and Fig. 3B). A similar pattern of expression is obtained with the positive control (RmEH). After four days in culture, the infected *T.ni*-H5 cells yielded almost five-fold more enzyme activity than the SF-21 cells (90 instead of 19 μmol.min^{-1}.l^{-1}). The specific activity (Fig. 3C and Fig. 3D) increased with time, and both infected cell lines reached almost the same epoxide hydrolase activity (70 nmol.min^{-1}.mg^{-1}) after five days. This value is one hundred times the specific activity obtained with the Lac Z virus, and illustrates not only the high quantity of Ms-JHEH produced using the baculovirus expression system (especially with the *T.ni*-H5 cells) but the low background of EH activity present in these cells compared to other eucaryotic expression systems (Grant et al., 1996). From a five day old culture of *T.ni*-H5 cells, the subcellular localization of the recombinant enzyme

was determined (Table 1). As expected, almost all the Ms-JHEH activity (90% of the total recovered) was found in the microsomal fraction. No activity was found in the cytosolic fraction. SDS/PAGE (Fig. 4A) of microsomal fractions shows a dense doublet of bands around 50 kDa (arrow level), as expected, from the cells expressing the Ms-JHEH (lane 3). This doublet did not appear in microsomes from cells infected with the Lac-Z virus (lane 4) or in the microsomal fraction from eggs of *M. sexta* (lane 2). A heterogeneous post-translational modification of the initial protein could have generated the doublet observed (O'Reilly et al., 1992). Using photolabeling (Fig. 4B), the microsomal preparation that contained the recombinant Ms-JHEH was labeled at the expected position (50 kDa) by EBDA (lane 1) and EFDA (lane 4) while no obvious labeling was shown for the microsomes with Lac Z gene (lane 2 and 3). Based on the intensity of labeling by EFDA, it seems that the lower band of the doublet at 50 kDa was the one more
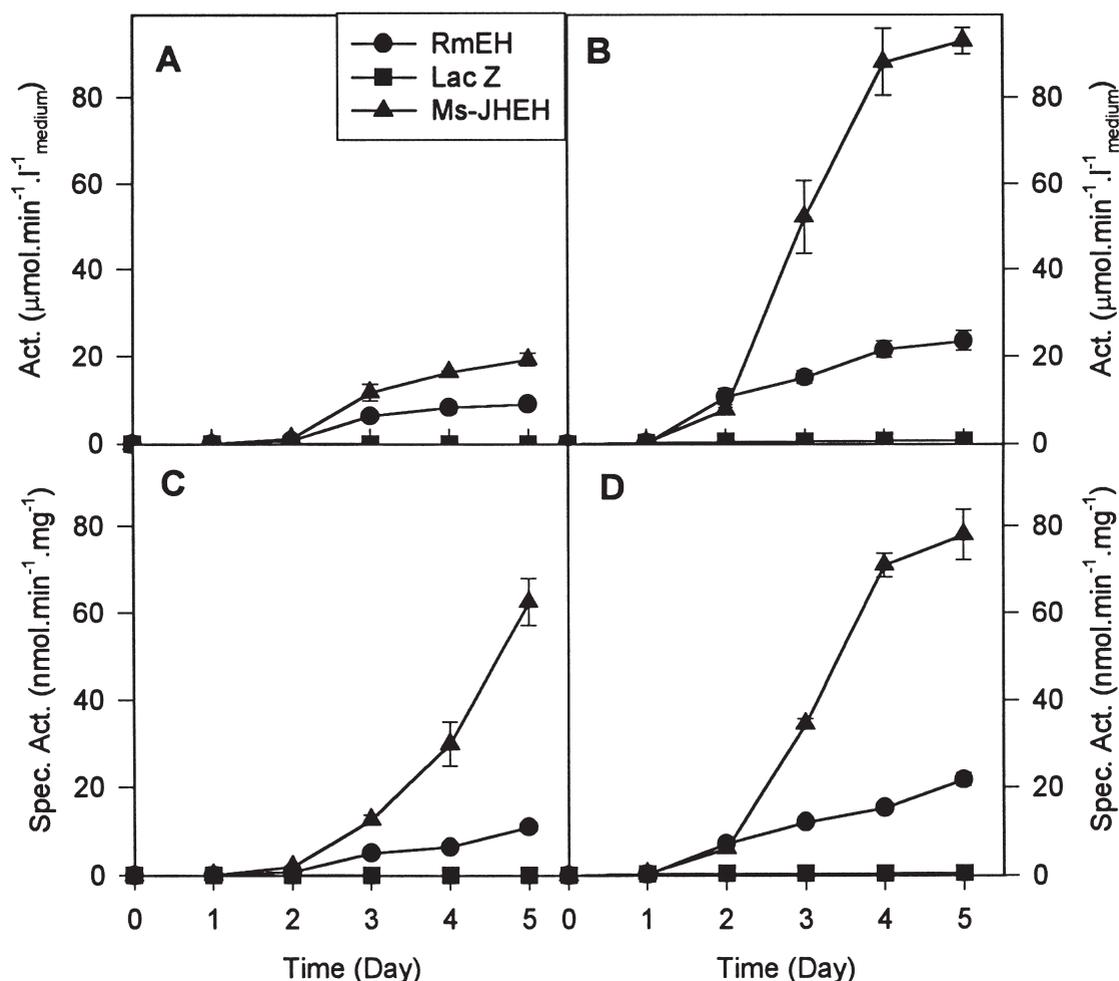

Fig. 3.   Kinetics of expression of epoxide hydrolase activity: Comparison of activity production in SF-21 (A) and *T.ni* H-5 (B) cells as a function of time. Changes in the specific activity in SF-21 (C) and *T.ni* H-5 (D) cells as a function of time. ■: control (Lac Z viruses); ●: positive control (RmEH); ◆: Ms-JHEH. Activity was measured with JH-III for Lac Z and Ms-JHEH, and c-SO for RmEH.

Table 1
Subcellular localization of the Ms-JHEH expressed in *T. ni* H5 cells. The preparation was from 200 ml of five day old culture

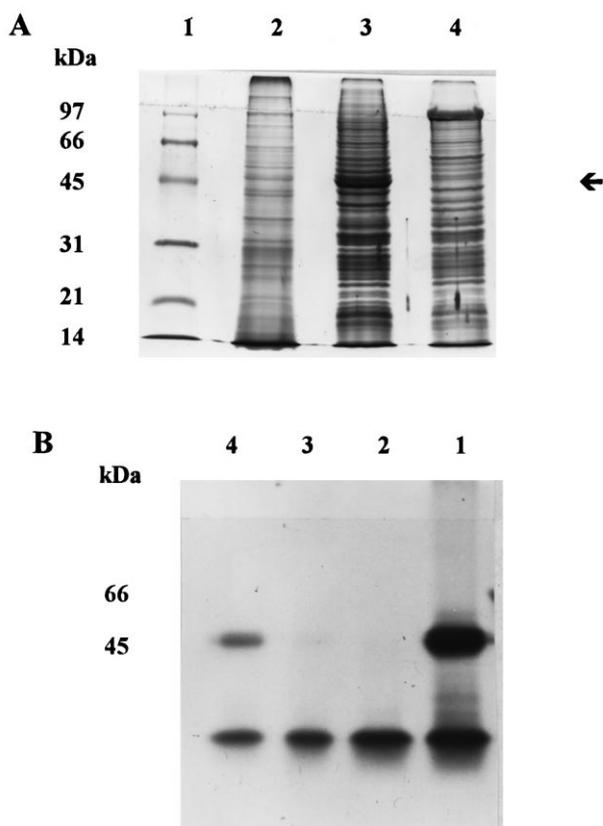|	Total act. (U)	Yield(%)	Spec. act. (mU.mg^{-1})	Purification fold
Crude Extract	32.5	100	133.7	1.0
Mitochondria Peroxisomes	2.3	7	48.5	–
Microsomes	24.5	76	659.2	4.9
Cytosol	0.6	2	4.5	–

1 U: 1 μmol.min^{-1} of diol produced from JH-III.

Fig. 4. A: SDS-polyacrylamide gel electrophoresis of crude microsomal fractions. *Lane 1*: molecular size marker; *lane 2*: eggs of *M. sexta* (12 μg); *lane 3*: *T. ni*-H5 cells infected with recombinant Ms-JHEH virus (20 μg); *lane 4*: *T. ni*-H5 cells infected with Lac-Z virus (20 μg). Arrow shows mobility of EH proteins. B: fluorogram of a SDS-PAGE of microsomal fractions of *T. ni*-H5 cells infected with recombinant Ms-JHEH virus labeled by EBDA (*lane 1*) or by EFDA (*lane 4*); and infected with Lac-Z virus labeled by EBDA (*lane 2*) or by EFDA (*lane 3*).

heavily labeled. Some nonspecific labeling was also obtained at the dye front. The photoaffinity labeling of Ms-JHEH by EBDA and EFDA suggests its affinity for molecules resembling the structure of JH (Prestwich et al., 1994; Wojtasek and Prestwich, 1996).

3.3. Comparison with the wild type enzyme

A microsomal fraction from eggs of *M. sexta* (egg extract) was prepared as described by Touhara and Prestwich (1993) for comparison with the recombinant enzyme. At first, activities of both extracts were measured for six substrates. The two enzymes showed a very similar spectrum of activity (Fig. 5). Microsomal fractions and the recombinant enzyme differed only in their ability to hydrolyze the epoxystearic acid. For the juvenile hormones, maximum activity was found for the JH-III, as expected (Touhara and Prestwich, 1993). For DPPO and SO the enzymes are more active on *trans* isomers than *cis*, the converse of the mammalian microsomal EH, which is more active on *cis* isomers (Borhan et al., 1995b).
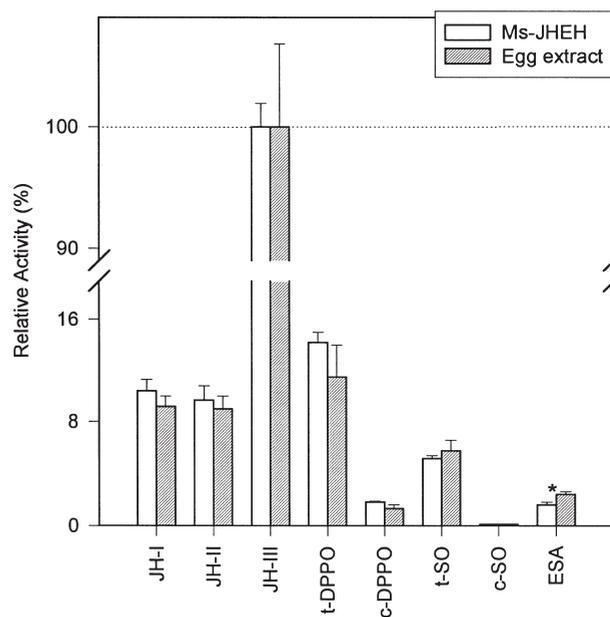

Fig. 5. Relative epoxide hydrolase activity for various substrates. The ratios were calculated from the specific activity obtained for the JH-III. The values are means ± S.D. (n = 3). Activities were measured using microsomal preparations from eggs of *M. sexta* and day 5 Ms-JHEH infected *T. ni* H5 cells. The specific activities for JH-III are 1.6 and 91.8 nmol.min^{-1}.mg^{-1} for the egg's extract and the Ms-JHEH, respectively.

In a second comparison, effects of different inhibitors were tested on the activity of both enzymes. The two enzymes showed very similar effects for all inhibitors tested (Fig. 6). Significant differences ($\alpha = 0.05$) were found only for mercuric chloride and (2S,3S)-para-nitro-phenyl-glycidol (SS-NPG). Ms-JHEH is more sensitive to trichloropropene oxide (TCPO), a microsomal EH inhibitor, than SS-NPG, chalcone oxide (CO) and 4-phenyl-chalcone oxide (4-PCO), soluble EH (sEH) inhibitors (Wixtrom and Hammock, 1985; Dietze et al., 1993a). Such a result agrees with the microsomal nature of Ms-JHEH. An inhibitor of metalloenzymes (EDTA) as well as an inhibitor of serine esterases and proteases (PMSF) showed no inhibition of the enzyme. A reducing reagent (dithiothreitol: DTT) and sulfhydryl-modifying reagent (iodoacetamide) did not have a significant effect on the activity of Ms-JHEH while HgCl$_2$ did. In contrast, the oxidant mCPBA was a potent inhibitor of Ms-JHEH activity. 5,5′-Dithio-nitrobenzoic acid (DTNB) has some effect on the enzyme activity. Similar results were obtained for mammalian microsomal EH (Wixtrom and Hammock, 1985). ω-Bromo-nitro-acetophenone (ω-Br-NPA) which binds covalently a histidine residue of the active site of mammalian mEH (Dubois et al., 1978) and sEH (Dietze et al., 1993b), only partially inhibited the activity of Ms-JHEH. Since a catalytic histidine responsible for activating water has been unequivocally demonstrated in other microsomal and soluble epoxide hydrolases (Hammock et al., 1997) and is uniformly present in all known members of the a/b hydrolase fold family of

proteins (Ollis et al., 1992). It is likely that the conserved histidine acts as a general base. The partial inhibition of Ms-JHEH by ω-Br-NPA was thus unexpected and may simply reflect difficulty with the compound reaching the catalytic histidine or turn-over of the alkylated enzyme. In esterases (another α/β fold enzyme class) the catalytic serine attacks the carbonyl of the ω-Br-NPA before the bromine is displaced by the histidine. Possibly, in Ms-JHEH the catalytic Asp and His are not in the correct orientation for this process to be efficient. Directed mutagenesis of His residues, as it was done for mammalian EH (Bell and Kasper, 1993; Pinot et al., 1995; Arand et al., 1996), will facilitate testing the involvement of a His residue in the enzyme mechanism.

3.4. PH optimum and precipitation of the enzyme-substrate intermediate

The activity of the recombinant enzyme was tested over a range of pH's from 4 to 10 (Fig. 7, black symbols). Maximum enzyme activity was obtained at pH 8.5–9.0, a value slightly different from the one determined (8.0) by Touhara and Prestwich (1993) for the JHEH from *M. sexta* eggs. At pH 7.0, there was approximately 50% of the maximum of activity. This result is consistent with the involvement of a histidine (pKa of 6.5 for the imidazole group) in the catalytic site of the Ms-JHEH.

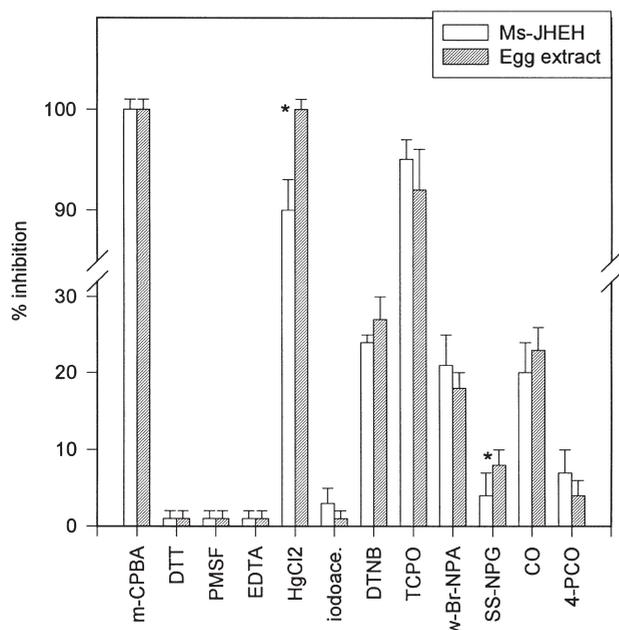We precipitated the intermediate between the enzyme

Fig. 6. Effect of various inhibitors on the activity of recombinant Ms-JHEH and extract from eggs of *M. sexta*. 2 mU of enzyme activity from each microsomal preparation was incubated 15 min with 1 mM inhibitor at 30°C; the remaining activity was then tested using JH-III as substrate. The values are means ± S.D. (n = 3).
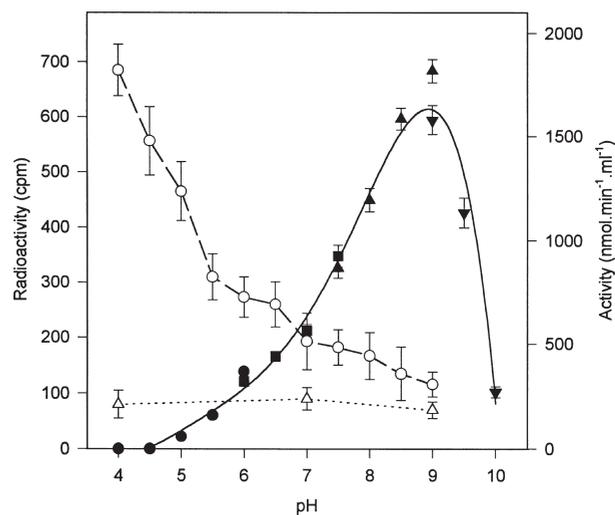

Fig. 7. Effect of the incubation pH on the enzyme activity (black symbols) of the recombinant Ms-JHEH and on its binding of [^{3}H] c-DPPO (white symbols). Activities were measured in ammonium acetate buffer (300 mM pH 4 to 6; •), sodium phosphate buffer (100 mM pH 6 to 7.5; ■), Tris/HCl buffer (100 mM pH 7.5 to 9; ◇) and ammonium chloride buffer (100 mM pH 9 to 10; ◆) using the conditions described in material and methods. 2.4 μg of crude Ms-JHEH was precipitated in presence of [^{3}H] c-DPPO for different pH (O) as described above. Background radioactivity in the precipitate was determined with 100 μg.ml^{-1} BSA (Δ).

and substrate (c-DPPO) as described for the mammalian EHs (Hammock et al., 1994; Müller et al., 1997). c-DPPO was chosen because it appeared to be a substrate turned over slowly by the enzyme (Fig. 5). Results are shown in Fig. 7 (white symbols). For the Ms-JHEH, maximum radioactivity bound was found at pH 4 and decreased with increasing pH to be almost the background level at pH 9.0. These results agreed with the action of a histidine in the catalytic mechanism of the Ms-JHEH as it is for the sEH (Hammock et al., 1994). At pH 4, increased radiolabeled protein (from 300 to 1300 cpm precipitated) was found with an increase in enzyme concentration (1.2 to 24 μg/tube).

4. Discussion

JH modulates a variety of developmental and physiological processes in insects (Gilbert and King, 1973). Previous studies suggest that hydrolytic enzymes play an important role in regulation of JH titer (Hammock, 1985; Roe and Venkatesh, 1990). Compared to juvenile hormone esterase, there is little known about JHEH. Indeed, JHEH may be an essential regulatory enzyme and could be applied to control insects (Hammock et al., 1988). To date, the only JHEH purified has been from eggs of *M. sexta* (Touhara and Prestwich, 1993).

We report the first expression in vitro of a microsomal epoxide hydrolase from an insect encoded by a cDNA. Using a baculovirus expression system, the recombinant protein Ms-JHEH was produced at a high level, especially in *T.ni* H5 cells. The selected recombinant virus SDCM1 represents a useful molecular tool for the preparation at large scale of Ms-JHEH to investigate both its biochemical and structural properties as well as its role in JH metabolism.

The recombinant protein Ms-JHEH presents biochemical properties similar to mammalian mEH: (i) the enzyme has a molecular mass of approximately 50 kDa, (ii) the optimum pH for the enzyme activity was found to be between pH 8.5 and 9, and (iii) the enzyme catalysis was not sensitive to DTT or iodoacetamide but was somewhat sensitive to DTNB and ω-BrNPA. However, in contrast to mammalian microsomal EHs, Ms-JHEH hydrolyses *trans* isomers (t-DPPO, t-SO) faster than *cis* isomers (c-DPPO, c-SO). This property is more like the mammalian and plant soluble EHs (Borhan et al., 1995b). Interestingly the soluble mammalian EH was discovered while studying mammalian metabolism of juvenoids. In contrast to the mammalian microsomal EHs which fail to hydrolyze trisubstituted epoxides such as squalene oxide and JH, the mammalian soluble enzyme rapidly metabolizes JH and juvenoids (Hammock et al., 1974; Mumby and Hammock, 1979b). The presence of more than one EH in the crude microsomal fraction of eggs could explain the small difference

observed in its ability to hydrolyze the epoxy-stearic acid compared to the expressed Ms-JHEH (Fig. 5).

The high sequence identity and similarity of Ms-JHEH to mammalian mEHs (44% identity and 64% similarity to human mEH) (Wojtasek and Prestwich, 1996) suggest that these proteins could derive from a common ancestral gene and could share similar biological functions. In *Manduca sexta*, Ms-JHEH may be implicated both in detoxification processes like the mammalian EHs and in the regulation of JH titer. Based on inhibition and substrate selectivity there appear to be at least three insect microsomal EHs. One degrades JH, one degrades certain cyclodiene insecticides, and one degrades monosubstituted and probably 1,2-disubstituted epoxides (Mullin and Wilkinson, 1980b).

The hydrolysis activity of Ms-JHEH toward JH-III was 10 fold more efficient than hydrolysis of JH-I and JH-II despite the fact that only the two latter are the natural substrates present in eggs of *M. sexta*. These results are in agreement with those found by Touhara and Prestwich (1993). A similar relative enzyme activity on JH homologs was observed with the murine soluble epoxide hydrolase (Hammock et al., 1974). It is likely that several factors influence the slower rate with higher JH homologs. One factor is that greater steric hindrance could retard both the attack of the nucleophilic aspartic acid on the JH epoxide and the subsequent attack of activated water on the resulting hydroxy-alkyl intermediate. Also the increased hydrophobicity of covalent enzyme adducts of JH-I and II are likely to lead to slow dealkylation. This hydrophobic effect on dealkylation and deacylation is common among enzymes of the α/β hydrolase fold family. Kinetic data support dealkylation as the rate limiting step of hydrolysis with the microsomal EH of mammals (Tzeng et al., 1996).

Little is known about the catalytic mechanism of insect EHs. For Ms-JHEH, the conservation of all residues proposed to form the catalytic triad in human mEH (Wojtasek and Prestwich, 1996) suggest that Ms-JHEH may utilize the same mechanism of epoxide hydration as mammalian EHs (Lacourciere et al., 1993; Hammock et al., 1994), shown by covalent labeling to involve an acyl-enzyme intermediate. In Ms-JHEH, the catalytic triad may correspond to Asp [227]-His [428]-Asp [350]. Our preliminary results on catalytic mechanism of Ms-JHEH suggest the participation of a histidine residue and the formation of a putative hydroxyl-alkyl enzyme intermediate. Consequently, a hypothetical scheme for the catalytic mechanism in insect mEH is proposed (Fig. 8). In the first step of the enzymatic reaction, the reactive nucleophile Asp [227] attacks the oxirane ring at the least hindered carbon atom leading to a ring opening and the formation of an hydroxyl-alkyl enzyme. In the second step of the reaction this covalent intermediate is hydrolyzed by a water molecule that is activated by the His [428]-Asp [350] pair. This mechanism is consistent with
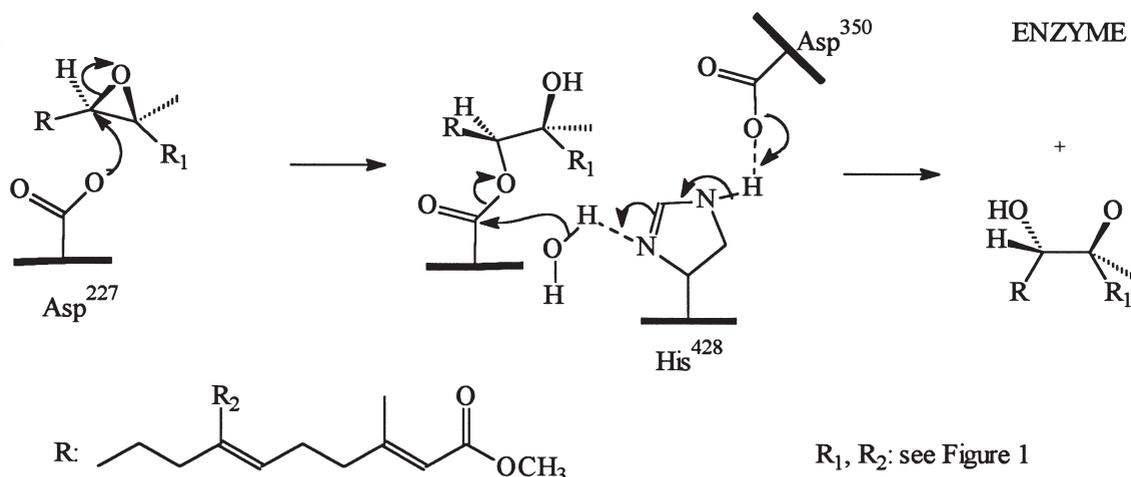
Fig. 8.   Proposed mechanism of action of the JHEH of *M. sexta*.

the *cis*-epoxide of JH-I yielding a threo diol with inversion at the C-10 carbon and retention at the C-11 carbon during the process (Grieneisen et al., 1995).

Finally, the Ms-JHEH baculovirus will also form a useful tool for analyzing the effects of the in vivo expression of JHEH in insects. In this context, the recombinant virus SDCM1 may be an effective way to reduce JH titers in vivo and this represents an attractive genetically engineered insecticide.

Acknowledgements

References

Arand, M., Wagner, H., Oesch, F., 1996. Asp[333], Asp[495] and His[523] form the catalytic triad of the rat soluble epoxide hydrolase. J. Biol. Chem 271, 4223–4229.

Bell, P.A., Kasper, C.B., 1993. Expression of rat microsomal epoxide hydrolase in *Escherichia coli*. Identification of a hystidyl residue essential for catalysis. J. Biol. Chem 268, 14011–14017.

Borhan, B., Jones, A.D., Pinot, F., Grant, D.F., Kurth, M.J., Hammock, B.D., 1995a. Mechanism of soluble epoxide hydrolase: formation of an α-hydroxy ester-enzyme intermediate through Asp[333]. J. Biol. Chem 270, 26923–26930.

Borhan, B., Mebrahtu, T., Nazarian, S., Kurth, M.J., Hammock, B.D., 1995b. Improved radiolabeled substrates for soluble epoxide hydrolase. Anal. Biochem 231, 188–200.

Borhan, B., Nazarian, S., Stocking, E.M., Hammock, B.D., Kurth,

M.H., 1994. An efficient preparation of [¹⁸O]-labeled epoxides. J. Org. Chem 59, 4316–4318.

Campbell, P.M., Healy, M.J., Oakshott, J.G., 1992. Characterization of juvenile hormone esterase in *Drosophila melanogaster*. Insect Biochem. Mol. Biol 22, 665–677.

Casas, J., Harshman, L.G., Hammock, B.D., 1991. Epoxide hydrolase activity on juvenile hormone in *Manduca sexta*. Insect Biochem 21, 17–26.

Dietze, E.C., Casas, J., Kuwano, E., Hammock, B.D., 1993a. Inhibition of epoxide hydrolase from human, monkey, bovine, rabbit and murine liver by *trans*-3-phenylglycidols. Comp. Biochem. Physiol 104B, 309–314.

Dietze, E.C., Stephens, J., Magdalou, J., Bender, D.M., Moyer, M., Fowler, B., Hammock, B.D., 1993b. Inhibition of human and murine cytosolic epoxide hydrolase by group-selective reagents. Comp. Biochem. Physiol 104B, 299–308.

Dubois, G.C., Appella, E., Levin, W., Lu, A.Y.H., Jerina, D.M., 1978. Hepatic microsomal epoxide hydrase: involvement of a histidine at the active site suggests a nucleophilic mechanism. J. Biol. Chem 253, 2932–2939.

Gilbert, L.I., King, D.S., 1973. Physiology of growth and development: endocrine aspsts. In: Rockstein, M. (Ed.), The Physiology of Insects. Academic Press, New-York, pp. 249–370.

Gill, S.S., Ota, K., Hammock, B.D., 1983. Radiometric assays for mammalian epoxide hydrolases and glutathione-S-transferase. Anal. Biochem 131, 273–282.

Grant, D.F., Greene, J.F., Pinot, F., Borhan, B., Moghaddam, M.F., Hammock, B.D., McCutchen, B., Ohkawa, H., Luo, G., Guenthner, T.M., 1996. Development of an in situ toxicity assay system using recombinant viruses. Biochem. Pharmacol 51, 503–515.

Grieneisen, M.L., Kieckbush, T.D., Mok, A., Dorman, G., Latli, B., Prestwich, G.D., Schooley, D.A., 1995. Characterization of the juvenile hormone epoxide hydrolase (JHEH) and juvenile hormone diol phosphotransferase (JHDPT) from *Manduca sexta* Malpighian tubules. Arch. Insect Biochem. Physiol 30, 255–270.

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

Hammock, B.D., 1985. Regulation of juvenile hormone titer. In: Kerkut, G.A., Gilbert, L.I., (eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology. Pergamon Press, Oxford, pp. 431–472.

Hammock, B.D., Harshman, L.G., Philpott, M.L., Székács, A., Ottea, J.A., Newitt, R.A., Wroblewski, V.J., Halarnkar, P.P., Hanzlik,

T.N., 1988. Strategies for the discovery of insect control agents: Exploitation of biomechanisms regulating insect development. In: Biomechanisms Regulating Growth and Development, Steffens, G.L., Rumsey, T.S. (eds.), USDA Beltsville Symposia in Agricultural Research vol. 12, Kluwer Academic Publishers, Dordrecht. pp. 137–173.

Hammock, B.D., Gill, S.S., Casida, J.E., 1974. Synthesis and morphogenetic activity of derivatives and analogs of aryl geranyl ether juvenoids. J. Agric. Food Chem 22, 379–385.

Hammock, B.D., Grant, D., Storm, D., 1997. Chapter 18: Epoxide Hydrolases. In: Sipes, I., McQueen, C., Gandolfi, A., (eds.), Comprehensive Toxicology. Pergamon Press, Oxford, pp. 283–306.

Hammock, B.D., Pinot, F., Beetham, J.K., Grant, D.F., Arand, M.E., Oesch, F., 1994. Isolation of a putative hydroxyacyl enzyme intermediate of an epoxide hydrolase. Biochem. Biophys. Res. Commun 198, 850–856.

Harshman, L.G., Casas, J., Dietze, E.C., Hammock, B.D., 1991. Epoxide hydrolase activities in *Drosophila melanogaster*. Insect Biochem 21, 887–894.

Lacourciere, G.M., Armstrong, R.N., 1993. The catalytic mechanism of microsomal epoxide hydrolase involves an ester intermediate. J. Am. Chem. Soc 115, 10466–10467.

Lacourciere, G.M., Vakharia, V.N., Tan, C.P., Morris, D.I., Edwards, G.H., Moos, M., Armstrong, R.N., 1993. Interaction of hepatic epoxide hydrolase derived from recombinant baculovirus expression system with an azarene oxide and an aziridine substrate analogue. Biochem 32, 2610–2616.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature 227, 680–685.

Lassiter, M.T., Apperson, C.S., Roe, R.M., 1995. Juvenile hormone metabolism during the fourth stadium and pupal stage of the Southern House Mosquito *Culex quinquefasciatus*. J. Insect Physiol 41, 869–876.

Mohamed, M.A., Lerro, K.A., Prestwich, G.D., 1989. Polyacrylamide gel miniaturization improves protein visualization and autoradiographic detection. Anal. Biochem 177, 287–290.

Müller, F., Arand, M., Frank, H., Seidel, A., Hinz, W., Winkler, L., Hänel, K., Blée, E., Beetham, J.K., Hammock, B.D., Oesch, F., 1997. Visualization of a covalent intermediate between microsomal epoxide hydrolase, but not cholesterol epoxide hydrolase, and their substrates. Eur. J. Biochem 245, 490–496.

Mullin, C.A., 1988. Adaptive relationships of epoxide hydrolase in herbivorous arthropods. J. Chem. Ecol 14, 1867–1888.

Mullin, C.A., Wilkinson, C.F., 1980a. Purification of an epoxide hydrolase from the midgut of the Southern Army Worm (*Spodoptera eridania*). Insect Biochem 10, 681–691.

Mullin, C.A., Wilkinson, C.F., 1980b. Insect epoxide hydrolase: properties of a purified enzyme from the Southern Army Worm (*Spodoptera eridania*). Pest. Biochem. Physiol 14, 192–207.

Mumby, S.M., Hammock, B.D., 1979a. A partition assay for epoxide hydrases acting on insect juvenile hormone and an epoxide-containing juvenoid. Anal. Biochem 92, 16–21.

Mumby, S.M., Hammock, B.D., 1979b. Substrates selectivity and stereochemistry of enzymatic epoxide hydration in the soluble fraction of mouse liver. Pestic. Biochem. Physiol 11, 275–284.

Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., Silman, I., Schrag, J., Sussman, J.L., Vershueren, K.H., Goldman, A., 1992. The α/β hydrolase fold. Prot. Eng 5, 197–211.

O'Reilly, D.R., Miller, L.K., Luckow, V.A., 1992. Baculovirus expression vectors: a laboratory manual. W.H. Freeman and Company, Oxford, England.

Pinot, F., Grant, D.F., Beetham, J.K., Parker, A.G., Borhan, B., Landt, S., Jones, A.D., Hammock, B.D., 1995. Molecular and biochemical evidence for the involvement of the Asp-333-His-523 pair in the catalytic mechanism of soluble epoxide hydrolase. J. Biol. Chem 270, 7968–7974.

Prestwich, G.D., Robles, S., Wawrzenczyk, C., Bühler, A., 1987. Hemolymph juvenile hormone binding proteins of lepidopterous larvae: enantiomeric selectivity and photoaffinity labeling. Insect Biochem 17, 551–560.

Prestwich, G.D., Singh, A.K., Carvalho, J.F., Koeppe, J.K., Kovalick, G.E., 1984. Photoaffinity labels for insect juvenile hormone binding proteins: synthesis and evaluation in vitro. Tetrahedron, Symposium-in Print 40, 529–537.

Prestwich, G.D., Touhara, K., Riddiford, L.M., Hammock, B.D., 1994. Larva lights: a decade of photoaffinity labeling with juvenile hormone analogues. Insect Biochem. Molec. Biol 24, 747–761.

Roe, R.M., Kallapur, V., Linderman, R.J., Viviani, F., Harris, S.V., Walker, E.A., Thompson, D.M., 1996. Mechanism of action and cloning of epoxide hydrolase from the cabbage looper *Trichoplusia ni*. Arch. Insect Biochem. Physiol 32, 527–535.

Roe, R.M., Venkatesh, K., 1990. Metabolism of juvenile hormones: degradation and titer regulation. In: Rutgers, G.A. (Ed.), Morphogenetic Hormones of Arthropods, vol. 1. University Press, New Brunswick, pp. 126–179.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Nat Acad. Sci. USA 74, 5463–5467.

Touhara, K., Prestwich, G.D., 1993. Juvenile hormone epoxide hydrolase: photoaffinity labeling, purification and characterization from Tobacco Hornworm eggs. J. Biol. Chem 268, 19604–19609.

Tzeng, H.-F., Laughlin, L.T., Lin, S., Armstrong, R.N., 1996. The catalytic mechanism of microsomal epoxide hydrolase involves reversible formation and rate-limiting hydrolysis of the alkyl-enzyme intermediate. J. Am. Chem. Soc 118, 9436–9437.

Ujvári, I., Eng, W.-S., Prestwich, G.D., 1990. Syntheses of homologous, tritium-labeled analogs of the natural juvenile hormones JH-I JH-II, and JH-III. J. Label. Cmpnd. Radiopharm 28, 65–72.

Wing, K.D., Sparks, T.C., Lovell, V.M., Levinson, S.O., Hammock, B.D., 1981. The distribution of juvenile hormone esterase and its interrelationship with other proteins influencing juvenile hormone metabolism in the cabbage looper *Trichoplusia ni*. Insect Biochem 11, 473–485.

Wisniewski, J.R., Rudnicka, M., Kochman, M., 1986. Tissue specific juvenile hormone degradation in *Galleria mellonella*. Insect Biochem 16, 843–849.

Wixtrom, R.N., Hammock, B.D., 1985. Membrane-bound and soluble-fraction epoxide hydrolases: methodological aspects. In: Zakim, D., Vessey, D.A. (Eds.), Biochemical Pharmacology and Toxicology, vol. 1. John Wiley and Sons, New York, pp. 1–93.

Wojtasek, H., Prestwich, G.D., 1996. An insect juvenile hormone-specific epoxide hydrolase is related to vertebrate microsomal epoxide hydrolases. Biochem. Biophys. Res. Comm 220, 323–329.