Identification and regulation of the juvenile hormone esterase gene in the Colorado potato beetle

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Contents

List of abbreviations

Ac	*Autographica californica*
α-NA	α-naphthyl acetate
bp	basepair
cDNA	complementary DNA
CPB	Colorado potato beetle
Dp-1	diapause protein-1
IEF	isoelectric focusing
JH	juvenile hormone
JHBP	juvenile hormone binding protein
JHE	juvenile hormone esterase
kb	kilobase
kDa	kilodalton
LD	long day
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
RP-HPLC	reversed phase-high pressure liquid chromatography
RT	reverse transcription
SDS	sodium dodecyl sulfate
SD	short day
Vg	vitellogenin

Amino acids:

Ala(A)	alanine
Arg(R)	arginine
Asn(N)	aspargine
Asp(D)	aspartate
Cys(C)	cysteine
Gln(Q)	glutamine
Glu(E)	glutamate
Gly(G)	glycine
His(H)	histidine
Ile(I)	isoleucine
Leu(L)	leucine
Lys(K)	lysine
Met(M)	methionine
Phe(F)	phenylalanine
Pro(P)	proline
Ser(S)	serine
Thr(T)	threonine
Trp(W)	tryptophan
Tyr(Y)	tyrosine
Val(V)	valine

Nucleotides:

A:	adenine
C:	cytosine
T:	thymine
G:	guanine

Stellingen

1. Voor de expressie van diapauze-eiwitten in de Coloradokever is het noodzakelijk dat eerst het juveniel hormoon esterase (JHE) tot expressie komt.
    Dit proefschrift.

2. Het JHE van de Coloradokever is opgebouwd uit twee subeenheden van gelijke grootte, dit in tegenstelling tot JHE's bij vlinderachtigen die uit één subeenheid bestaan.
    Dit proefschrift.

3. De conclusie dat het eiwit 'ultraspiracle' (USP) functioneert als nucleaire receptor voor juveniel hormonen is voorbarig.
    Jones and Sharp (1997) *Proc. Natl. Acad. Sci. USA* 94: 13499-13503.
    Feyereisen (1998) *Proc. Natl. Acad. Sci. USA* 95: 2725-2726.

4. De elementen 'cryptochrome' (CRY) en 'timeless' (TIM) van de dagritmeklok bij insekten, zijn beide goede kandidaten om tevens daglengte bij te houden.
    Science (1998) 282: 2157.
    Dunlap (1999) *Cell* 96: 271-290.

5. Bij het ontwikkelen van een malariavaccin zou meer aandacht besteed moeten worden aan het mechanisme dat verantwoordelijk is voor natuurlijke gastheer-immuniteit.
    Ramasamy (1998) *Biochim. Biophys. Acta* 1406: 10-27.
    Fried *et al.* (1998) *Nature* 395: 851-852.
    Holder (1999) *Proc. Natl. Acad. Sci. USA* 96: 1167-1169.

6. Nederlanders zouden een voorbeeld kunnen nemen aan Japanners wat betreft het wachten in een rij.

7. Overdreven hygiëne is slecht voor de gezondheid.

8. Beter een duveltje in een glas dan uit een doosje.

Stellingen behorend bij het proefschrift 'Identification and regulation of the juvenile hormone esterase gene in the Colorado potato beetle'.

Wageningen, 6 april 1999
A.M.W. Vermunt

CHAPTER 1

General introduction

Juvenile hormone esterase

Juvenile hormone esterase (JHE) is an enzyme, which degrades juvenile hormone (JH) in insects (Roe & Venkatesh, 1990; de Kort & Granger, 1996). This specific esterase catalyzes the hydrolysis of the ester group in JH (Fig. 1a) into the biologically inactive JH-acid (Hammock, 1985). JH maintains the larval stage of insects. That is why it is named juvenile hormone. A drop in the JH titer is necessary for initiation of metamorphosis (Riddiford, 1994). In the adult stage of many insects, high JH titers are required for reproduction, whereas low JH titers lead to an arrested development (Wyatt & Davy, 1996). Drops of JH are caused by a decline in JH biosynthesis and a rise of JHE activity (de Kort & Granger, 1996). The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is an excellent model organism for studies on the regulation of JH titer, because drastic changes in the JH titer can be induced by environmental factors such as daylength (photoperiod) (de Wilde *et al.*, 1968; de Kort *et al.*, 1982; de Kort, 1990).
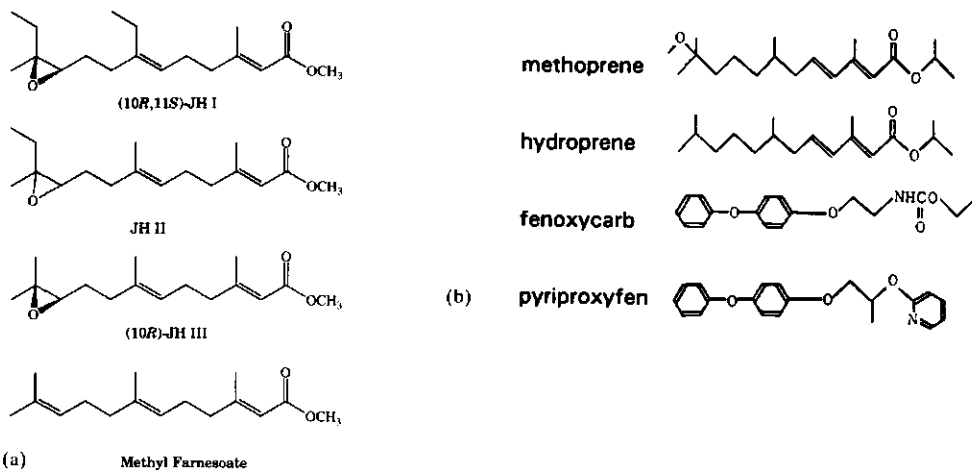

Fig. 1 (a) Chemical structures of natural juvenile hormones (JHs) found in insects. JH III is the major JH in the Colorado potato beetle. (b) Juvenile hormone analogs that are used as insect growth regulators.

Colorado potato beetle

The Colorado potato beetle is an important pest species in potato culture. Especially in some parts of the United States it is difficult to control this pest. Due to overuse of a wide range of insecticides this beetle developed resistance to many of these pesticides (Casagrande, 1987). In the Netherlands, the beetle was a major agricultural pest in the middle of this century. This was one of the reasons why research on the Colorado potato beetle was initiated at the Department of Entomology of the Wageningen Agricultural University (de Kort, 1990). Understanding the physiology of feeding and reproduction, and the ecology of the Colorado potato beetle, may lead to improved pest management strategies. To approach agricultural problems insight needs to be obtained in the fundamental biological question: "What are the underlying mechanisms of behavioral responses to environmental stimuli?" As a result of recent research efforts, a considerable amount of information has become available about the physiology and behavior of the Colorado potato beetle, for example how it adapts to its environment for survival. This knowledge and that of other species were used to develop new and more selective insecticides, like the insect growth regulators (IGR's) methoprene, fenoxycarb and pyriproxyfen (Fig. 1b; Hammock & Quistad, 1981; Staal, 1982; Itaya, 1987; Grenier & Grenier,1993).

The Colorado potato beetle is a member of the family Chrysomelidae (leaf beetles). The species originates from southern Mexico. From there it spread around the Americas. It became a major pest when it adapted to cultivated potato. It was probably imported with potato into Europe. The beetles mainly feed and deposit their eggs on leaves of potato or on close relatives of potato in the plant family Solanaceae (Hsiao, 1988). Reasons for the wide geographic distribution are the general availability of host plants and its high fecundity. Individual females lay between 300 and 800 eggs (Harcourt, 1971).

Under laboratory conditions, the life cycle of the Colorado potato beetle is well studied. The larvae hatch from the egg 5 days after oviposition and undergo three larval instars of 2-3 days each. At day 6 of the 4th larval instar, the larvae stop feeding and dig into the soil to pupate. Eleven to twelve days after digging, it emerges from the soil as an adult. Depending on the daylength, adults will reproduce or enter diapause. Beetles reared under long-day conditions (16 h light : 8 h dark), show reproductive behavior and females start depositing their eggs five days after adult emergence. Under short-day conditions (10 h light : 14 h dark) beetles enter diapause 10-12 days after adult emergence by digging into the soil (de Kort et al., 1982; 1997).

Seasonal adaptation

Diapause is an adaptive phenomenon that permits the beetle to survive during unfavorable environmental conditions, such as extreme temperatures or lack of water or food. Reduced activity and cessation of feeding and reproduction characterize the diapause behavior. Typical for diapause is that environmental programming normally occurs far in advance of the unfavorable conditions. The insect anticipates the period of adversity and is already fully prepared when the adverse condition actually arrives (Adkinson, 1966; Denlinger, 1985). Two different types of seasonal adaptation in *Leptinotarsa* beetles are known. In temperate regions, short daylength is the environmental trigger to induce winter diapause. Here, diapause is terminated by a rise in temperature. In warmer and arid regions diapause is induced by food shortage or quality loss of host plants (de Wilde *et al.*, 1969; Saunders, 1982; Hsiao, 1988). Diapause results not only in enhanced survival during unfavorable environmental conditions, but also increases the mating chance through synchronization of development of males and females in a population (Denlinger, 1985; Yamashita, 1996).
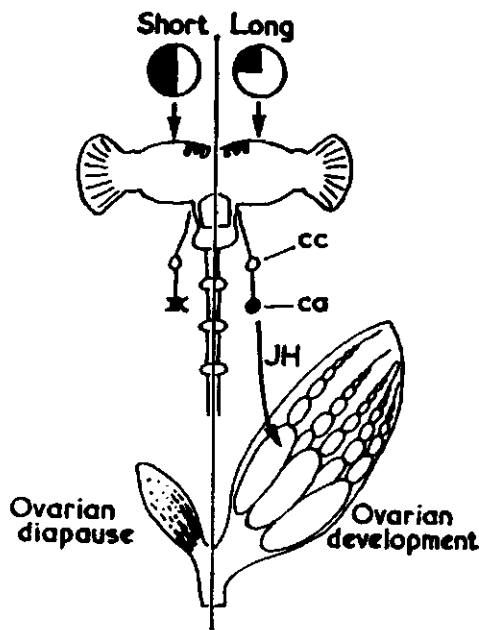
Fig. 2. Schematic diagram of diapause in the Colorado potato beetle (after de Wilde, 1959; Saunders, 1982). Short days stimulate the neurosecretory cells to release peptides, which are transported along axons and the corpora cardiaca (cc) and inhibit juvenile hormone (JH) synthesis in the corpora allata (ca). The resulting low titer of juvenile hormone causes yolk synthesis for development of the oocytes to cease. Long days induce ovarian development.

Signal transduction pathway of photoperiod-induced diapause

Winter diapause in the Colorado potato beetle is induced by a photoperiod of shorter than 15 h light per day at 25°C (de Kort, 1990). The photoperiodic perception occurs in the head of the beetle, but not by the eyes (de Wilde et al., 1959). The photoreceptor for the photoperiodic clock is probably located in the median part of the brain in insects, but the identity of the receptor and the clock are yet unknown (Takeda & Skopik, 1997). Carotenoids are thought to be involved in photoreception for diapause induction in insects and mites, but the exact function of carotenoids in this process is not yet clear (Veerman et al., 1983). Some kind of photoperiodic time measurement has to take place in the brain to stimulate the medial and lateral neurosecretory cells. These cells can produce allatostatins and allatotropins, neuropeptides which, respectively, inhibit or stimulate the corpora allata in the head. These neuropeptides are transported along axons and released near the corpora allata (Khan, 1988; Stay et al., 1994). Active corpora allata synthesize juvenile hormone (JH) (Figs 1, 2). It is a drop of this hormone, which mediates the photoperiod-induced diapause. JH application to pre-diapausing, non-reproductive adults can reverse the diapause resulting in reproductive animals. Diapause can also be induced by removal of the corpora allata of the beetle (allatectomy). Allatectomy of long-day females leads to a decrease in JH titer of the hemolymph, which results in cessation of oviposition followed by digging behavior at the onset of diapause. Thus, photoperiodic induction of diapause is mediated by JH (de Wilde & de Boer, 1961).
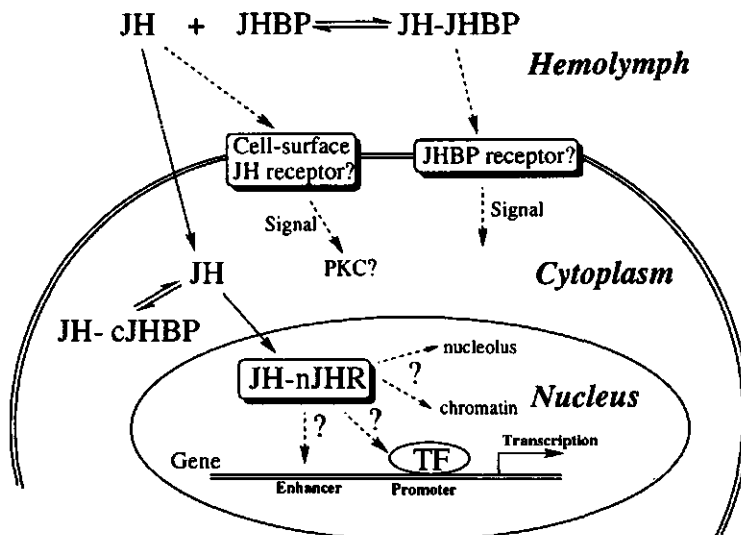

Fig. 3. JH signal transduction (after Prestwich et al., 1994). The mode of transit of JH across the cell membrane is as yet unknown, and the mechanism by which a JH-nJHR complex suppresses or activates transcription genes is also unknown. JH, juvenile hormone; JHBP, JH binding protein; c, cytosolic; n, nuclear; JHR, JH receptor; PKC, protein kinase C; TF, transcription factor.

The concentration of JH at its target organs is pivotal for its morphogenetic and gonadotrophic effects (Fig. 2). Several juvenile hormones (JHs) have now been identified. They are sesquiterpenoid molecules (Riddiford, 1994). The major JH in the Colorado potato beetle is JH III (methylepoxyfarnesoate; Fig. 1) (de Kort et al., 1982). JH molecules are chemically related to the vertebrate terpene all-*trans* retinoic acid (RA), which regulates development in vertebrates (Gudas et al., 1994). In contrast to RA, the molecular mechanism of action of insect JH is far from established. Probably, JH acts on membrane receptors (Ilenchuk & Davey, 1987; Yamamoto et al., 1988; Sevala et al., 1995; Webb & Hurd, 1995) as well as on nuclear receptors (Riddiford, 1994; Forman et al., 1995)(Fig. 3).

Jones and Sharp (1997) found that JH binds to the nuclear receptor ultraspiracle (USP). They proposed that JH binds to USP and induce conformational changes to form homo-oligomers, which can regulate transcription of genes. USP is also involved in forming a hetero-dimer with the ecdysone receptor. This hetero-dimer, when stabilized by ecdysone, binds to ecdysone responsive elements of ecdysis- and metamorphosis-related genes to switch these genes on or off (Thomas et al., 1993; Yao et al., 1993). In contrast to binding to a nuclear receptor, a membrane receptor-mediated effect of JH involves calcium and protein kinase C (Yamamoto et al., 1988). Protein kinase C regulates both phosphorylation and dephosphorylation of transcription factors, like *jun* and *fos*, which may form hetero-dimers with steroid hormone receptors and in turn regulate gene transcription (Riddiford, 1994; Karin, 1991).

The presence or absence of JH at target organs leads to a cascade of activation and inactivation of several genes resulting in the onset of a certain developmental program. In the adult Colorado potato beetle, high JH titers in the hemolymph result in a reproductive program. For instance, the vitellogenin gene in females is activated by JH and its product is necessary for oocyte development (de Kort et al., 1997). In contrast, low JH titers lead to the disappearance of vitellogenins, but also to the synthesis of enormous amounts of storage proteins in preparation for diapause (de Kort et al., 1997).

Regulation of the juvenile hormone titer
The JH titer in the hemolymph is mainly determined by the rate at which juvenile hormones are synthesized and released by the corpora allata on one hand and by the degradation of JH through specific hydrolases on the other hand. These hydrolases are juvenile hormone esterase (JHE) which is mainly active in the hemolymph and juvenile hormone epoxide hydrolase (JHEH) which is mainly active in cellular microsomes (Fig. 4; Hammock, 1985; Roe & Venkatesh, 1990; Prestwich et al., 1994; de Kort & Granger, 1996). JH is hydrophobic and to reach its target organs it is carried by a juvenile hormone binding protein (JHBP). JHBPs are present in the hemolymph and in the cells of target organs. They also protect JH from degradation by general carboxyl esterases and facilitate specific degradation by JHE (Fig. 4; Prestwich et al. 1994; de Kort & Granger, 1996).
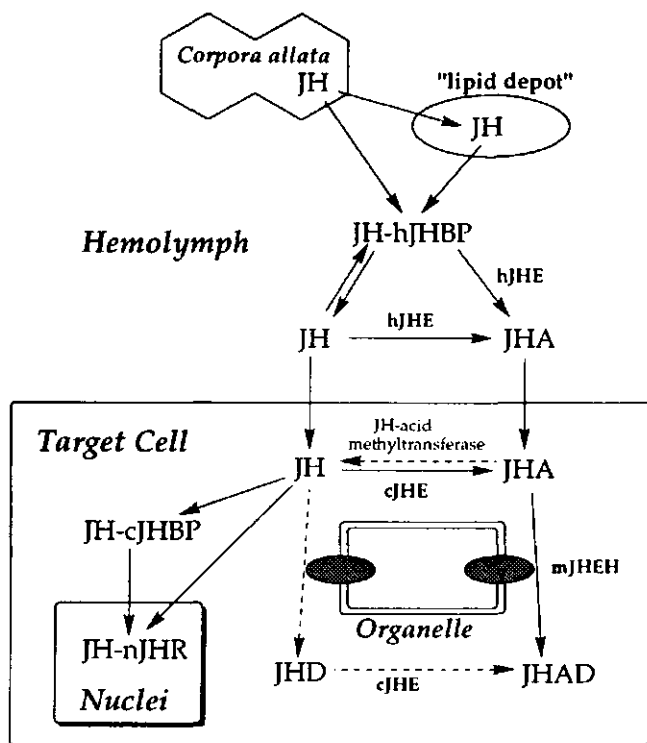
Fig. 4. Schematic summary of JH transport, metabolism, and action (after Prestwich *et al.*, 1994). h, hemolymph; c, cytosolic; m, membrane-associated; n, nuclear; JHBP, JH binding protein; JHR, JH receptor; JHE, JH esterase; JHEH, JH epoxide hydrolase; JHA, JH acid; JHD, JH diol; JHAD, JH acid diol.

Among insects, the regulation of the JH titer has been most intensively studied in Lepidoptera. A summary of endocrine events in lepidopteran larvae during the fourth and fifth instar is illustrated In Figure 5 (Touhara *et al.*, 1995). Larval ecdysis is induced by ecdysteroids in the presence of JH. A decrease in synthesis of JH and a rise in JHE lead to lower JH titers, which are necessary for initiation of metamorphosis. A relationship between JH and JHE levels has been observed for two peaks of JHE activity during the last larval instar: the prewandering and the prepupal peak (Fig. 5; Roe & Venkatesh, 1990).
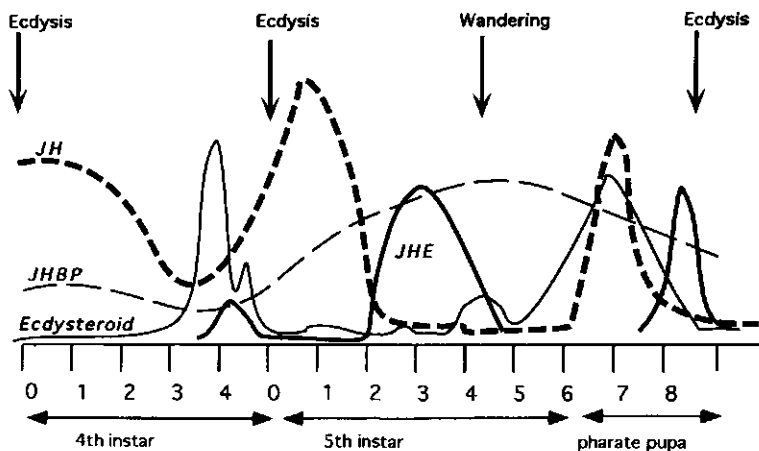
Fig. 5. Schematic diagram of endocrine events occurring in lepidopterous larva during (after Touhara *et al.*, 1995). Days of each instar are indicated on the horizontal axis. The vertical scale for each titer is different. JH, juvenile hormone; JHBP, JH binding protein; JHE, JH esterase. Days of each instar are indicated on the horizontal scale.

The JHE peak, which precedes the onset of adult diapause in the Colorado potato beetle (Kramer *et al*, 1976), probably serves the same function as the prewandering peak in lepidopteran larvae. The JHE peak coincides with a decrease in corpora allata activity (Khan *et al.*, 1982; de Kort, 1990). Both are necessary for the removal of JH in the hemolymph in order to induce diapause.

Juvenile hormone esterase in the Colorado potato beetle.
The regulation of the JH titer in the Colorado potato beetle has been studied by Kramer *et al.* (1976a, 1976b, 1977, 1978a, 1978b) and by Khan *et al.* (1982a, 1982b). The studies of Kramer, concerning JHE, involved the developmental profile, biochemical properties and regulation of JHE activity. JHE activity in the beetle can be distinguished from general carboxyl esterase activity by its resistance to the potent serine esterase inhibitor diisopropylfluorophosphate (DFP) and by its sensitivity to the non-ionic detergent Triton X-100. Treatment of diapausing animals with JH, enhanced JHE activity. This enhancement could be prevented by inhibition of translation or transcription, suggesting regulation of the JHE activity at those levels.

In this thesis, the JHE protein and its gene are characterized and its regulation in relation to metamorphosis and diapause of the Colorado potato beetle is investigated.

13

Outline of thesis

The research described in this thesis focuses on the molecular characterization of juvenile hormone esterase (JHE) from the Colorado potato beetle. This information is necessary to address the following questions:

- In which physiological processes is JHE involved?
- What is the structure of the protein?
- What is the structure of the encoding gene and how is its expression regulated?
- When and where is it synthesized during development?
- What is the effect of JH and daylength on the expression of the JHE gene?
- How is JHE gene expression regulated in the diapause program?

To answer these fundamental questions we followed a biochemical and molecular genetic approach.

In **Chapter 2**, the JHE activity in the hemolymph of the Colorado potato beetle during its life cycle is investigated. From the stage with the highest activity, the JHE enzyme was purified and characterized. Cloning and sequence analysis of the putative encoding gene based on cDNA analysis is described in **Chapter 3**. **Chapter 4** provides evidence for the existence of two JHE-related genes in the Colorado potato beetle. A genomic DNA analysis of these genes was also performed. Expression of the putative JHE gene *in vivo* is described in **Chapter 5**, in which also effects of a JH analog and photoperiodic treatment of the beetle on JHE enzyme activity and JHE-mRNA levels are reported. Attempts to express the putative JHE genes in a baculovirus-insect cell system to demonstrate the functionality of recombinant JHE are described in **Chapter 6**. In **Chapter 7** the relation between different JH-dependent genes (JHE, Diapause protein-1 and Vitellogenin) of the adult Colorado potato beetle is discussed. Finally, in **Chapter 8** the results are summarized and the role of JHE in the process of signal reception to gene expression and from protein function to behavior is addressed.

CHAPTER 2

Purification and characterization of juvenile hormone esterase from hemolymph of the Colorado potato beetle

A.M.W. Vermunt, A.M.G. Vermeesch and C.A.D. de Kort
Archives of Insect Biochemistry and Physiology (1997) 35, 261-277

ABSTRACT

In the Colorado potato beetle (*Leptinotarsa decemlineata*), low juvenile hormone (JH) titers are necessary to initiate metamorphosis and diapause. Low JH titers coincide with high activities of JH esterase, which occur mainly in the hemolymph. The specific activity of JH esterase appeared to be highest in the last larval instar, at day 3 after the moult and reached a value of 13.5 nmol/min/mg. JH esterase was purified from hemolymph collected at this stage by a sequence of separation systems including preparative nondenaturing PAGE, isoelectric focusing and SDS-PAGE. The enzyme had a molecular weight of 120,000 and was composed of two subunits with molecular weights of 57,000. The subunits were not linked by disulphide bridges. Isoelectric focusing revealed two forms of the enzyme with isoelectric points of 5.5 and 5.6. The K_m and k_{cat} of the purified enzyme were determined. The major form with a pI 5.6 had a K_m of 1.4×10^{-6}M and a k_{cat} of 0.9 s^{-1} and the minor form with a pI 5.5 had a K_m of 2.2×10^{-6}M and a k_{cat} of 1.9 s^{-1}. The quaternary structure of *L. decemlineata* JH esterase as a dimer differs from JH esterases in other species, which are monomers.

INTRODUCTION

Juvenile hormone (JH) plays a key role in the endocrine control of molting, metamorphosis and reproduction (Riddiford, 1994; Wyatt & Davy, 1996; de Kort & Granger, 1996). It has been established for the Colorado potato beetle, *Leptinotarsa decemlineata*, that JH also mediates day-length related phenomena and that JH titers differ greatly between adult beetles preparing for diapause or for reproduction (de Kort, 1990). It is known that JH titers are controlled by JH biosynthesis in the corpora allata and by JH degradation (Kramer, 1978a; Hammock, 1985; Roe & Venkatesh, 1990; de Kort & Granger, 1996). JH degradation in the Colorado potato beetle is known to occur mainly in the hemolymph by JH specific esterases. The activity of these esterases seems to vary greatly in the course of adult life (Kramer & de Kort, 1976a). An intriguing finding was that the JH esterase activity was high in conditions where the JH titer was low such as during short-day photoperiods leading to diapause and low when JH titers were high during ovary maturation in long-day conditions (de Kort, 1990). It seems that the function of this enzyme is to clear JH from the hemolymph in stages where JH should be at a low level for certain short-day-induced processes to take place properly. This makes it worthwhile to study JHE in more detail in order to comprehend the role of this enzyme in day-length-induced phenomena.

The present study focuses on the isolation of JHE from the hemolymph of the Colorado potato beetle and the characterization of the enzyme in physico-chemical terms. For this purpose it was first necessary to determine first the developmental stage in which the enzyme activity is highest. Enzyme activities were measured throughout larval and adult development and in both long-day and short-day rearing conditions. The data thus obtained are discussed in relation to those from other insect species. The next step will be the further structural analysis of the enzyme, followed by cloning the JHE gene and study its regulation.

MATERIALS AND METHODS

Insects
Larvae and adults of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), were reared in the laboratory under two different photoregimes (short-day and long-day conditions) as described previously (Koopmanschap *et al.*, 1992). Ages of specimens in the last larval (4th) instar are expressed in days after the molt to this instar. Ages of imaginal specimens are expressed in days after emergence from the soil, which is 1-2 days after the metamorphic molt.

Hemolymph collection
Hemolymph was collected in capillary pipettes from larvae or adults of well-defined age, by clipping a leg or wing. Tyrosinase activity was inhibited by the addition of a few crystals of phenylthiourea. Hemocytes were removed by centrifugation for 2 minutes at 15,000 g and the treated hemolymph was stored at -20°C.

Enzyme and protein assays

The JH esterase assay was performed according to the partition method of Hammock and Sparks (1977), as modified by Lefevere (1989), in 50 mM Tris-HCl, pH 8.0 as assay buffer. As substrate was used, racemic [^{3}H]-JH III (Amersham) mixed with unlabeled JH III (Calbiochem) at a final concentration of 5 x 10^{-6} M. Undegraded JH was extracted with isooctane. The aqueous phase with JH-acid was counted for radioactivity in a liquid scintillation counter (Beckman LS 6000TA). For determination of the Michaelis constant (K_m) triplicate Lineweaver-Burk plots were generated after incubation at 5 substrate concentrations in the range of 1 x 10^{-7} M to 5 x 10^{-6} M. The turnover number (k_{cat}) was calculated by using the v_{max} from the Lineweaver-Burk plots, the protein concentration and the molecular weight estimated from analytical nondenaturing PAGE.

The general carboxyl esterase assay was performed according to van Asperen (1962) with α-naphthyl acetate as substrate. The activity was measured spectrophotometrically at 600 nm after staining the hydrolysis product α-naphthol with diazoblue - sodium lauryl sulphate.

Protein concentrations were determined according to Bradford (1976) with the Bio-Rad protein assay (Bio-Rad) using bovine serum albumin as a standard. The determined specific activities of JHE were expressed in (nmol JH) / min / (mg total protein) per sample.

Electrophoresis

Nondenaturing PAGE was carried out on the Bio-Rad Protean II slab cell with 7.5% polyacrylamide gels in a continuous buffer system of 0.1 M glycine titrated with Tris to pH 8.3. Gels were run at a constant current of 20 mA for 1 hour. For nondenaturing PAGE with a gradient gel of 7-20% or 10-20% polyacrylamide, the LKB Multiphor electrophoresis system was used. Electrophoresis was performed with a constant voltage of 200 V for 20 hours in the same nondenaturing PAGE buffer as mentioned above.

SDS-PAGE was carried out according to Laemmli (1970) in 8.5% polyacrylamide vertical slab gels on the Bio-Rad Protean II slab cell. After electrophoresis, the proteins were either visualized or eluted for the JHE assay. JHE activity was localized in the nondenaturing gels after incubating serial gel slices of 2 mm in 200 ml 50 mM Tris-HCl pH 8.0 overnight at 4°C. Protein staining was done with 0.1% Coomassie Brilliant Blue R-250 in 40% methanol, 10% acetic acid, and gel destaining in 25% ethanol, 9% acetic acid.

Isoelectric focusing

Separation of proteins was also performed with a LKB Multiphor isoelectric focusing system. Hemolymph and purified samples were focused on precast wide (pH 3.5-9.5) and narrow pH range (pH 4.0-6.5) Ampholine PAGplates (5% polyacrylamide, 2.2% ampholytes; 1 mm thick) of Pharmacia. Samples were diluted with distilled water to a volume of 15 µl with 1% glycine. Focusing was performed according to the instructions of the manufacturer. The proteins in the gels were stained with Coomassie Brilliant Blue R-250 or eluted from 2 mm slices for enzyme assays.

Elution occurred by overnight diffusion at 4°C in 200 ml of 50 mM Tris-HCl buffer, pH 8.0. The eluate was assayed for JHE activity. For determination of the isoelectric points of proteins, the pI calibration Kit (pH 3-10) from Pharmacia was used.

Enzyme purification

Hemolymph from three days old 4th-instar larvae was used as enzyme source for purification of juvenile hormone esterase from the Colorado potato beetle. The first step in the purification was preparative nondenaturing PAGE with the Model 491 Prep Cell of Bio-Rad according to the instructions of the manufacturer, using a 37 mm ID glass tube with a 6 cm high, 5% polyacrylamide gel. The sample, 2 ml in total, containing 1.75 ml of hemolymph (28.6 mg protein), 0.01% bromophenol blue, 10% glycerol and 0.1 x nondenaturing PAGE buffer was loaded on the gel and electrophoresed in nondenaturing PAGE buffer (0.1 M glycine titrated with Tris to pH 8.3) at constant power of 5 W. Proteins were eluted at a rate of 0.5 ml/min. One hundred and twenty fractions of 1.0 ml were collected and analyzed for protein concentration, JHE activity and general carboxyl esterase activity. The fractions containing high JHE activity were pooled and concentrated in 30 K Macrosep tubes (Filtron) according to the instructions of the manufacturer. Samples of the concentrate were electrophoresed by nondenaturing PAGE (slab gel), SDS-PAGE and IEF for analysis. The second step of the purification was preparative isoelectric focusing using a precast IEF gel (Ampholine PAGplates) with a pH gradient of 4-6.5. The prepurified and concentrated sample from nondenaturing PAGE of 0.5 ml was loaded at the length of 14 cm of the gel, near and parallel to the cathode. After focusing, the gel was sliced in 2 mm wide serial strips (14 cm in length) in the area between pI marker 5.2 and 5.85 in which the JHE activity was focused in pilot experiments. The proteins were eluted from each slice by overnight diffusion at 4°C in 1.0 ml of 50 mM Tris-HCl buffer, pH 8.0. Samples from the eluates were assayed for JHE activity and protein concentration.

Electro-elution

Electro-elution of the electrophoresed and stained proteins was performed with a Model 422 Electro-Eluter from Bio-Rad according to the instructions of the manufacturer. Eluted samples were concentrated in a Speed Vac (Savant). SDS was removed by precipitation of the protein in 10% TCA followed by centrifugation. The protein pellet was washed twice with 100 ml of ice-cold acetone and air-dried. The protein pellet was redissolved in SDS-PAGE sample buffer and prepared for analysis with SDS-PAGE.

18

RESULTS

Specific activity of JHE throughout the life cycle

There are three developmental stages where JH titers are low and where chances are good to find relatively high JHE activities: the last larval instar, adults kept under short-day conditions and virgin females. The hemolymph of animals of different age were sampled to determine which precise developmental stage contains highest JHE activity.

Blood was collected from specimens throughout the 4th larval instar and the pupal stage. Both protein concentration and JHE activities were determined in duplicate from at least three samples for each time point, each of which was pooled hemolymph of 4-6 animals. Average values were calculated (Fig. 1). During the fourth larval instar the JHE activity increased till a peak activity on the third day of 13.5 nmol/min/mg protein and then it decreased until the 6th day, the time of digging into the soil. At the 9th day a second JHE peak was detected with a value of 12.4 nmol/min/mg. The peak coincides with the time of pupation, which occurs three days after digging into the soil (de Kort et al., 1982). The activities throughout the pupal stage were low.
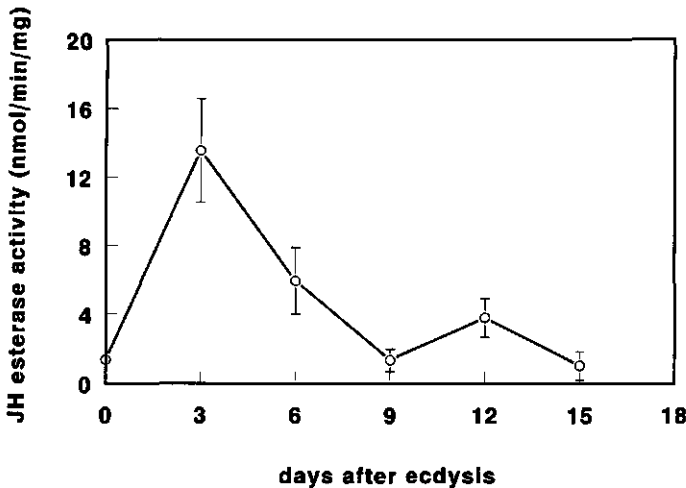

Fig. 1. Specific activity of JHE throughout the fourth larval instar and pupal stage. Data represent the JHE activity per mg protein in pooled hemolymph samples of 4-6 individuals and are the mean ±SD of samples collected from at least three independent rearings. At day 6, 4th-instar larvae dig into the soil and at day 9 they pupate.
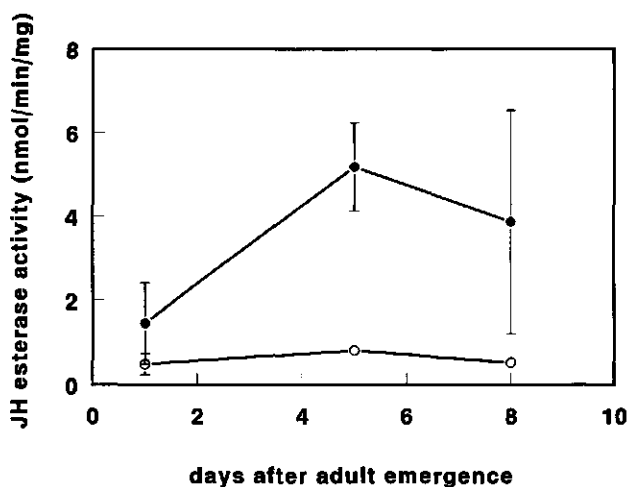
Fig. 2. Specific activity of JHE in adults of different age reared under short-day (●) and long-day conditions (○). Data represent the JHE activity per mg protein in pooled hemolymph samples of 4-6 individuals and are the mean ±SD of at least three independent rearings.

The hemolymph of adults (mixed sexes) kept under long-day (LD) or short-day (SD) for 1, 5 or 8 days was assayed for protein concentration and JHE activity (Fig. 2). Specific activity was low in beetles, 1 day after emergence from the soil, regardless the photoperiod. Higher activities were present in beetles of 5 days old and JHE activities were much higher in SD than in LD beetles. Maximum values were present in 5-day old SD beetles (5.2 nmol/min/mg protein). Yet, all values were lower than in 3-day-old 4th-instar larvae.

Females either mated or unmated produce a first batch of eggs between 4-6 days after emergence. Thereafter, egg production increases to a rate of 40-60 eggs per day in mated females, but virgin females reduce oviposition to rates as low as 0-10 eggs per day. It is supposed, but not yet proven, that JH levels in unmated females are relatively low (Dortland, 1979), and consequently, JHE levels may be high. Indeed, the specific JHE levels in virgin females of 2 weeks old and older were higher than in mated females (Fig. 3). However, here too, values are rather low compared to those found in the larval stages.

Comparing these data, we conclude that the best source for JHE isolation is the hemolymph from 3-day-old 4th-instar larvae, since the highest specific activity occurred at this developmental stage.
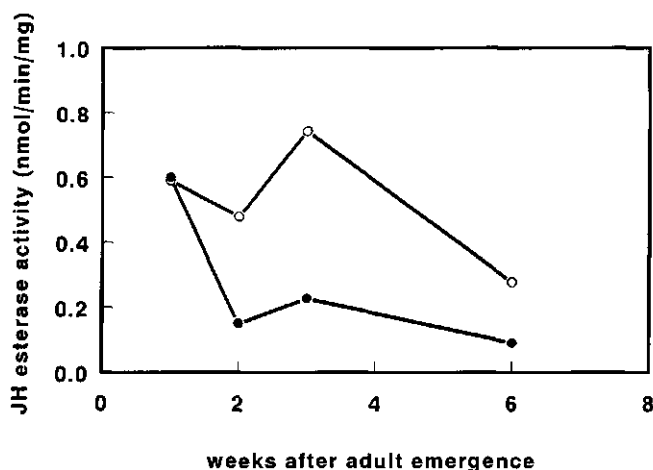
Fig. 3. Specific activity of JHE in virgin (o) and mated females (•). Data represent values of the JHE activity per mg protein in pooled hemolymph samples of 4-6 individuals.

Purification and characterization of JHE from 4th-instar larvae

The first purification step was preparative nondenaturing PAGE with the Prep Cell. The collection of fractions started after one hour, when the bromophenol blue eluted from the gel, and stopped after 120 fractions when UV readings from the detector were low. All fractions were analyzed for JHE activity, α-naphtyl acetate (α-NA) esterase activity and protein concentration (Fig. 4). Fractions 25-55 contained JHE activities with a maximum activity of 14.6 nmol/min/ml found in fraction 41. The α-NA esterase activity was detected in two sets of fractions: fractions 1-15 and fractions 80-110. Activities were higher in the first set (25.5 nmol/min/ml) than in the second set. Almost no overlap between JH and α-NA esterase activity was observed. Protein peaks were seen in fractions: 0-10, 20-30 and 65-80. JHE was purified from fractions 34-47 as these contained the highest specific activities and no α-NA esterase activity. Table 1 shows the results obtained from testing aliquots of original hemolymph and of nondenaturing PAGE eluate. The nondenaturing PAGE procedure gave a 41% recovery of the original JHE activity after 25-fold concentration of the eluate fractions (Table 1).
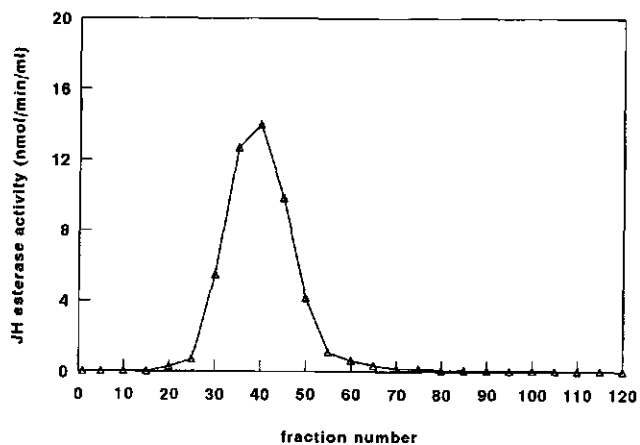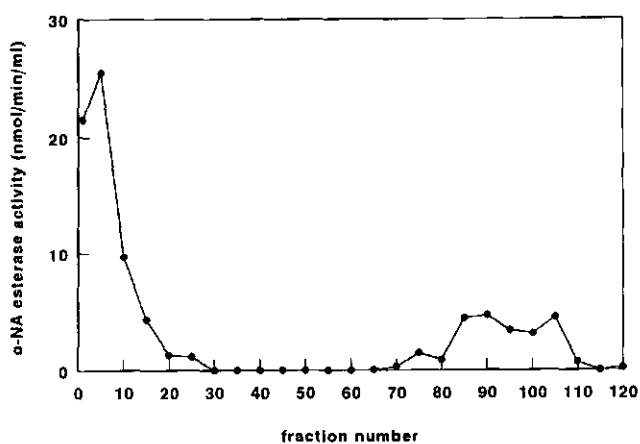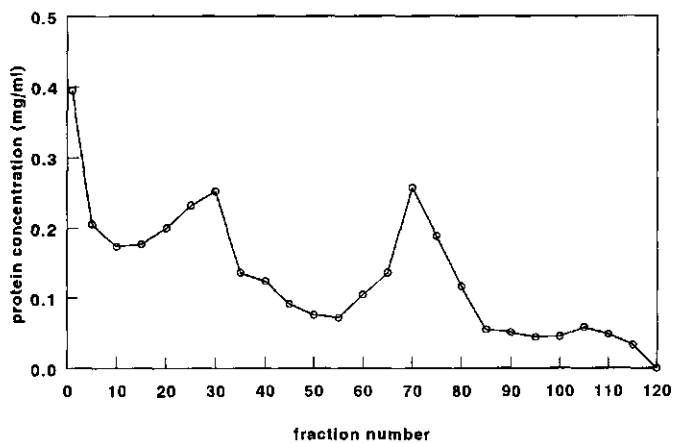
Fig. 4. Analysis of 1 ml fractions from hemolymph of 3-day-old 4th-instar larvae (1.75 ml), subjected to preparative nondenaturing PAGE using the Prep Cell.
(A) JHE activity; **(B)** α-Naphthyl acetate esterase activity; **(C)** Protein concentration.

Table 1. Purification by nondenaturing PAGE followed by IEF of JH esterase from the hemolymph of the Colorado potato beetle.

Material	Volume (ml)	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Yield (%)	Purification factor (fold)
Hemolymph	1.75	28.6	358	12.5	100	1.0
Native PAGE	0.50	1.6	147	91.8	41	7.5
IEF	4.00	0.043	79	1841	22	147

Hemolymph from 3-day-old 4th-instar larvae was used as the source for the purification. Nondenaturing PAGE was performed by using the Model 491 Prep Cell system. Fourteen 1 ml fractions from the Prep Cell with high activity were pooled and concentrated to 0.5 ml. After narrow isoelectric focusing on an IEF gel with a pH 4-6.5 gradient, the JH esterase was eluted from 2 mm-wide gel slices in 1 ml fractions.
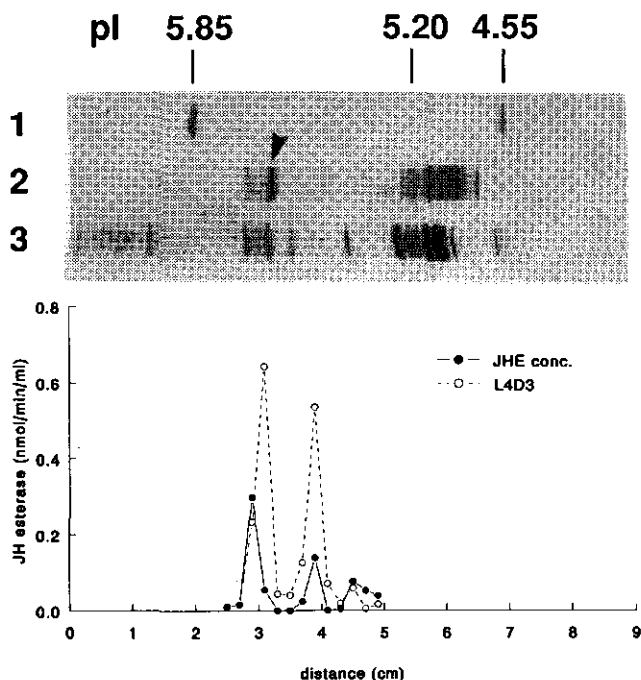
Fig. 5. Separation of proteins by isoelectric focusing in gel with narrow pH range (pH 4-6.5). Proteins were stained with Coomassie Brilliant Blue R-250.
Lane 1, pI markers; lane 2, JHE concentrate from pooled Prep Cell fractions 34-47, 15 µg of protein; lane 3, hemolymph of 3-day-old 4th-larval instar, 50 µg of protein.
The graph at the bottom represents JHE activity determined in the eluate of 2 mm slices of parallel lanes in the same experiment. Note that JHE in hemolymph appears in two isoforms.

The next step in the purification procedure was isoelectric focusing (IEF). Analytical IEF was performed for determination of the isoelectric point. IEF on a gel with a wide pH range (pH 3-10) indicated that all JHE activity of hemolymph samples from different stages was focused between pH markers 5.20 and 5.85 (data not shown). A narrow range IEF gel was used for a more accurate determination of the pI of JHE. On a pH 4-6.5 IEF gel, JHE activity of total hemolymph was focused at two positions: a major peak at pH 5.6 and a minor peak at pH 5.5 (Fig. 5). If the concentrated eluate from nondenaturing PAGE was subjected to narrow range IEF, a third but minor peak was observed at pH 5.4. For preparative purification purposes 0.5 ml of the concentrated eluate of the preparative nondenaturing PAGE was loaded on a pH 4-6.5 IEF gel. The JHE activity was measured after slicing of the gel and elution of the proteins. Of the loaded activity, 54% was eluted overnight in a total of four 1 ml fractions (Table 1). The rest of the activity was eluted in later fractions or was positioned elsewhere at the gel, or possibly that the enzyme became less active during isoelectric focusing. The JHE activity was mainly focused in two regions of 4 mm separated from each other by 4 mm of gel. At pI 5.6, 72% of the eluted activity was found and at pI 5.5, 28% (Table 2).

The kinetic parameters K_m and k_{cat} were determined for both isoforms of purified JHE. JHE at pI 5.6 had a K_m of 1.4 ($\pm$0.2) x 10^{-6}M and a k_{cat} of 0.9 s^{-1} and at pI 5.5, the enzyme had a K_m of 2.2 ($\pm$0.2) x 10^{-6}M and a k_{cat} of 1.9 s^{-1} (Table 2).

Table 2. Kinetic parameters of purified JH esterase from the hemolymph of the Colorado potato beetle.

pI JH esterase	Percentage eluted enzyme	K_m (mM)	k_{cat} (s^{-1})
5.5	28	2.2$\pm$0.2	1.9
5.6	72	1.4$\pm$0.2	0.9

The molecular weight of the JHE from the Colorado potato beetle was determined using continuous and gradient electrophoresis gels. Samples of the JHE concentrate from the preparative nondenaturing PAGE were subjected to nondenaturing PAGE for molecular weight determination, using slab gel electrophoresis. After electrophoresis, the position of JHE activity in the lane was localized by slicing the gel in 2 mm slices and analyzing each slice. The proteins in the slices were eluted by buffer. The determination was done in three different types of gels. In a 7% continuous polyacrylamide gel, the JHE activity coincided with a position corresponding to a molecular weight of 135,000. The determination of molecular weight with nondenaturing PAGE is more accurate, when a gradient gel is used. A 7-20% gradient polyacrylamide gel indicated a molecular weight of 109,000

and a 10-20% gradient a molecular weight of 130,000 (Fig. 6). In Figure 6, a protein band can be seen at the position of JHE activity in case of the sample of the JHE concentrate (arrow). The average molecular weight determined on the gradient gels was 120,000. The position of JHE from crude hemolymph samples after nondenaturing electrophoresis corresponds with a 5-10% higher molecular weight (Fig. 6). The higher estimate of the molecular weight in crude hemolymph is probably due to interactions with other proteins that could cause retardation in the gel.
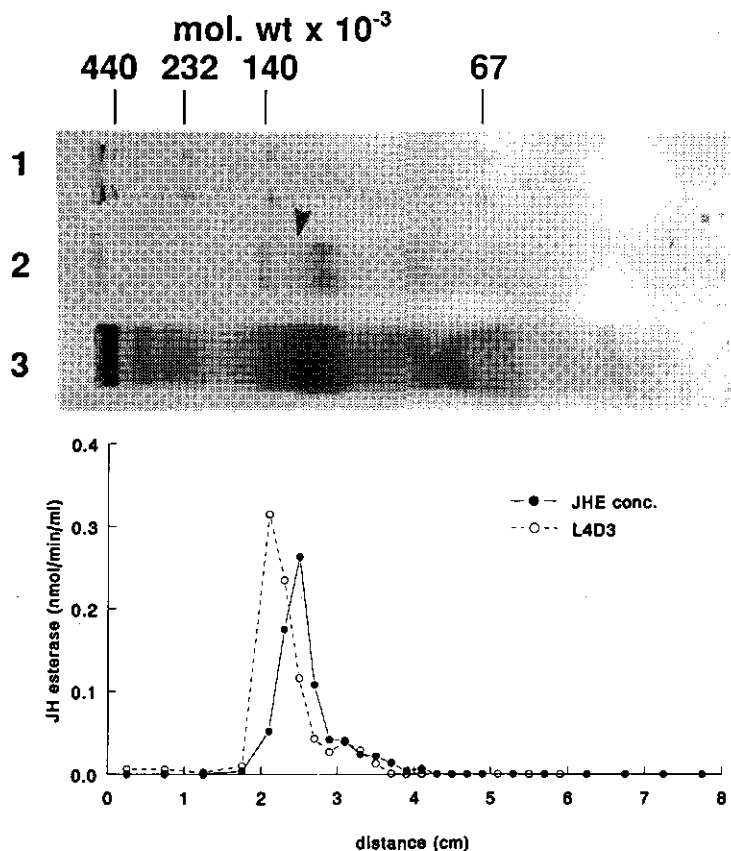
Fig. 6. Determination of molecular weight of JHE after separation on nondenaturing PAGE (10-20% gradient polyacrylamide gel). Proteins were stained with Coomassie Brilliant Blue R-250.
Lane 1, high molecular weight markers; lane 2, JHE concentrate from pooled Prep Cell fractions 34-47, 15 µg of protein; lane 3, hemolymph of 3-day-old 4th-instar larvae, 50 µg of protein. The graph at the bottom represents JHE activity determined in 2 mm gel slices of parallel lanes with JHE concentrate and hemolymph in the same experiment. Note that JHE activity in the concentrate corresponds with a molecular weight of 120,000 (arrow). JHE in hemolymph is slightly retarded due to interaction with other proteins.

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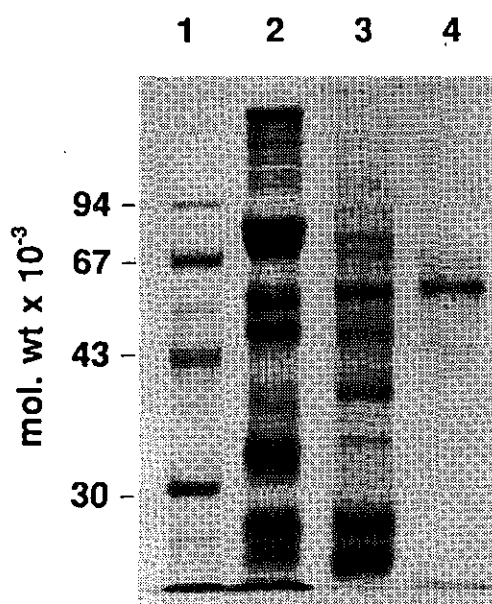
Fig. 7. Isolation of JHE subunit and determination of molecular weight of subunit. Proteins were separated by SDS-PAGE using 8.5% polyacrylamide gels and stained with Coomassie Brilliant Blue R-250.
Lane 1, molecular weight markers; lane 2, hemolymph of 3-day-old 4th-instar larvae, 30 μg of protein; lane 3, JHE concentrate from pooled Prep Cell fractions 34-47, 10 μg of protein; lane 4, protein recovered from isoelectric focusing band (pI 5.6) which was shown to contain JHE activity. The protein band in lane 4, which represents the JHE subunit, has a molecular weight of 57,000.

The proteins of the band on IEF gel, which coincided with JHE activity and pI 5.6 (arrow in Fig. 5), were electro-eluted and subsequently separated by SDS-PAGE. On gel, a strong protein band with a molecular weight of 57,000 was seen (Fig. 7, lane 4). The protein band of nondenaturing PAGE with a molecular weight of 120,000 which coincided with JHE activity (arrow in Fig. 6) also showed after electro-elution and SDS-PAGE a prominent protein band of 57,000 (data not shown). In the latter case, some minor protein bands were visible, which were the result of the incomplete separation of proteins on nondenaturing PAGE (Fig. 6). These results suggest that the purified pI 5.6 isoform of JHE with a native molecular weight of 120,000 is composed of two subunits. Unfortunately, the minor JHE IEF band(s) with pI 5.5 did not contain enough protein to determine its subunit composition. When a sample of the purified JHE with pI 5.6, eluted by diffusion from the IEF gel, was concentrated and subjected to SDS-PAGE, the 57,000 band appeared the most prominent. Other very minor protein bands were also seen, probably due to some curling of the pH gradient in the Ampholine PAGplates.

SDS-PAGE was also performed in the absence of β-mercaptoethanol in the sample buffer, to prevent the reduction of disulphide bridges if these exist between protein subunits. Under these conditions we again observed the presence of the 57,000 band (data not shown). This indicates that the two subunits of 57,000 were not linked by disulphide bridges.

DISCUSSION

JHE throughout the life cycle
The present study confirms and extends earlier observations about changes in JHE activity in the hemolymph of the Colorado potato beetle (Kramer & de Kort, 1976a). In adults, large differences in JHE activity of the hemolymph have been reported between long-day and short-day beetles. JHE activity, expressed per ml of hemolymph, showed a peak on day 8, if the beetles were kept under short-day conditions. The activity was at least 10 times higher than found in the hemolymph from long-day (reproductive) beetles. If the activity is expressed per mg of protein, the peak is observed on day 5 (Fig. 2). This can be explained by the fact that the JHE gene is expressed earlier than the genes for the major hemolymph proteins. Indeed, diapause proteins, which are the major components in the hemolymph of short-day beetles, increase in concentration mainly after day 6 (Dortland, 1978; Koopmanschap et al., 1992).

This study also extends earlier observations on JHE activity in larval hemolymph. We observed two peaks of JHE activity in the last larval instar and pupae (Fig. 1). The first peak on day 3 resembles the prewandering peak in the hemolymph of last instar larvae of Lepidoptera (de Kort & Granger, 1996), and is probably essential for the induction of digging behavior on day 6. The second peak, just around pupation, is very similar to the prepupal peak of JHE activity in Lepidoptera. The higher specific activity found in larvae is due to the lower concentration of the major hemolymph proteins compared with adults (de Kort & Koopmanschap, 1994; de Kort, 1996). Consequently, total protein concentration of the hemolymph is much higher in short-day adults than in last-instar larvae.

In addition to development and photoperiod, the present study demonstrated that mating also affects JHE activity. At the start of oviposition, between day 4 and 6, mated and unmated females showed similar JHE activity in the hemolymph. Subsequently, JHE activity decreased in mated females concomitant with an increase in rate of oviposition. In virgins, a drop in fecundity coincides with a significant increase in JHE activity of the hemolymph (Fig. 3). The difference between virgin and mated females would even be larger when the activity was expressed per ml hemolymph instead of per mg protein, because of the higher protein content in hemolymph of virgins. This suggests that JHE plays a more prominent role in JH titer regulation in virgins than in mated females. However, JHE activity in the virgin adults is much lower compared with last-instar larvae or short-day adults. Mating has distinct effects on JHE activity in different species. In two closely related moths, Choristoneura fumiferana and C. rosaseana, Cusson & Delisle

(1996) recently observed a significant increase in JHE activity in mated females relative to virgins on day 1, but no difference on days 3 and 5. However, in the cabbage looper, *Trichoplusia ni*, Venkatesh *et al.* (1988) reported results, which are similar to the situation in the Colorado potato beetle.

Differences in response between species can probably be explained by variation in the timing of copulation in relation to the overall oocyte cycle. In the Colorado potato beetle, vitellogenesis starts two days after adult emergence, whereas mating is observed between days 3 and 4. Therefore, mating is not required for the production of the first batch of eggs, but affects fecundity after the onset of oviposition.

Characterization of purified JHE

Several years ago, the presence of a distinct JHE in the hemolymph of the Colorado potato beetle was demonstrated by inhibition studies using diisopropyl fluorophosphate (Kramer and de Kort, 1976b). However, no attempts were made to purify this JHE. We have now demonstrated the purification and molecular characterization of this JHE by classical biochemical techniques using a combination of nondenaturing preparative gel electrophoresis, IEF and SDS-PAGE. Earlier attempts to purify this JHE by affinity chromatography using transition state inhibitors (Abdel-Aal & Hammock, 1986) were unsuccessful (A.M.W. Vermunt, personal observation).

By narrow range IEF, it appeared that two isoforms of the enzyme exist, a major form of pI 5.6 and a minor form with pI 5.5 (Table 2, Fig. 5). Both forms showed similar kinetic properties (Table 2). The K_m values for racemic JHIII of both forms are close to the values reported for crude hemolymph JHE (Kramer & de Kort, 1976b). In general, JHE from different species have K_m values of 10^{-7} to 10^{-6}M (Wing *et al.*, 1984; de Kort & Granger, 1996). The k_{cat} values for the Colorado potato beetle (Table 2) are similar to those of Lepidoptera, which vary between 1-2 s^{-1} (Hanzlik & Hammock, 1987; Ward *et al.*, 1992).

From the combinations of nondenaturing PAGE and SDS-PAGE or IEF and SDS-PAGE, in the presence or absence of β-mercaptoethanol, it appeared that JHE from the Colorado potato beetle is a dimer, in which the subunits are not linked by disulphide bridges (Figs 5, 6 and 7). The molecular weight of the enzyme was determined in two different nondenaturing gradient gels. The average value of these estimates was 120,000. Analysis of the same protein by SDS-PAGE revealed a band of 57,000, suggesting that this enzyme is composed of two subunits. Whether the dimer is a hetero- or homodimer remains to be established.

Comparison of JHEs in different species

The quaternary structure of JHE from *L. decemlineata* is clearly different from JHEs in other insect species, as a survey of the literature shows (Table 3). All purified Lepidopteran JHEs described so far are monomers with a molecular weight of 60,000-66,000 (table 3). Their isoelectric points are in the same range as JHE from *L. decemlineata*, and varied between pH 4.2 and 6.0. The quaternary structure of *L. decemlineata* JHE resembles acetylcholinesterase (AChE) from *L. decemlineata*,

which is a dimer of 130,000 (Zhu & Clark, 1994). But in contrast to JHE, AChE consists of subunits which are linked by disulphide bridges and its isoelectric point (pI 7.3) is almost two pH units higher.

Table 3. Comparison of the molecular structure of JH esterase from *L. Decemlineata* with several esterases from other insect species.

Insect species; enzyme	pI	Quaternary structure	M_r native	M_r subunits	reference
Leptinotarsa decemlineata JHE	5.6; 5.5	dimer	120 K	57 K	this study
Heliothis virescens JHE	4.8	monomer	61 K	61 K	Hanzlik *et al.*, 1989
Manduca sexta JHE	6.0; 5.5	monomer	66 K	66 K	Venkatesh *et al.*, 1990
Trichoplusia ni JHE	5.5; 5.4	monomer	64 K	64 K	Hanzlik & Hammock,1987
Lymantria dispar JHE	n.d.	monomer	62 K	62 K	Valaitis, 1992
Galleria mellonella JHE	5.0; 4.8	n.d.	60 K		Rudnicka & Kochman, 1984; McCaleb *et al.*, 1980
Gryllus rubens JHE	5.1; 4.2	n.d.	188 K		Zera *et al.*, 1992
Leucophaea maderae JHE	n.d	n.d.	47 K		Gunawan & Engelmann, 1984
Leptinotarsa decemlineata AChE	7.3	dimer S-S	130 K	65 K	Zhu & Clark, 1994
Drosophila melanogaster AChE	n.d	dimer S-S monomer	165 K 70 K	70 K	Gnagey *et al.*, 1987
Myzus persicae E4	n.d.	monomer	59 K	59 K	Field *et al.*, 1993
Culex quinquefasciatus Est	5.2	monomer	67 K	67 K	Ketterman *et al.*, 1992
Blatella germanica E6	4.8	monomer	55 K	55 K	Prabhakaran & Kamble, 1995

Abbreviations:M_r, molecular weight; JHE, juvenile hormone esterase; AChE, Acetylcholinesterase; the other esterases (E4, Est, E6) are general carboxyl esterases; S-S, presence of disulphide bridges between subunits; n.d., not determined; K, thousand.

Several indications exist that JHEs among insect species are structurally different, as determined by immunological and amino acid sequencing studies (Venkatesh et al., 1990; Valaitis, 1991). In addition, JHE from *L. decemlineata* was very sensitive to inhibition by Triton X-100, much more than Lepidopteran JHEs (Kramer & de Kort, 1976b). This sensitivity can possibly be explained by disaggregation of the JHE dimer subunits caused by the solubilization force of Triton X-100 resulting in inactive monomers.

Cloning and sequencing the JHE gene will eventually elucidate whether this esterase has evolved from some putative ancestral carboxyl esterase gene. The full amino acid sequence of JHE has so far only been determined from *Heliothis virescens* (Hanzlik et al., 1989) and partial sequences are known from *Manduca sexta* (Venkatesh et al., 1990), *Lymantria dispar* (Valaitis, 1991) and *Trichoplusia ni* (Venkataraman et al., 1994). The developmental- and tissue-specific expression of the JHE gene will provide further insight in how JHE activity is controlled and how the JH titer in turn is regulated in the programming of metamorphosis and in day-length dependent phenomena.

CHAPTER 3

Cloning and sequence analysis of cDNA encoding a putative juvenile hormone esterase from the Colorado potato beetle

A.M.W. Vermunt, A.B. Koopmanschap, J.M. Vlak and C.A.D. de Kort

ABSTRACT

In the Colorado potato beetle, *Leptinotarsa decemlineata*, reproduction and diapause are mediated by the juvenile hormone (JH) titer in the hemolymph. This titer is controlled by JH synthesis in the corpora allata and by JH degradation. The main pathway of JH degradation is by JH esterase in the hemolymph. The native JH esterase appeared to be a dimer consisting of two 57 kDa subunits (Vermunt *et al.*, 1997a). The 57 kDa subunit of JH esterase was digested with endoproteinase Lys-C and the digestion products were separated by reversed phase HPLC. Three different peptides were collected and sequenced. The amino acid sequence of one peptide showed high similarity to fragments of other insect esterases. Based on the amino acid sequence of these peptides, degenerate primers were constructed for RT-PCR. A PCR product of 1.3 kilobases was obtained and sequenced. This product was used to screen a cDNA library for a complete cDNA copy and to analyze the messenger RNA from larvae and adult beetles. The size of the messenger RNA was 1.7 kilobases. The complete amino acid sequence of the protein was deduced from the nucleotide sequence of overlapping clones from a cDNA library and a 5'RACE product. An open reading frame (ORF) of 1545 base pairs encoding the 57 kDa protein was characterized. The ORF contained the sequences of the three peptides. It showed no significant homology to other proteins present in databases, but it did contain several functional esterase motifs.

INTRODUCTION

The Colorado potato beetle belongs to those insect species in which juvenile hormone (JH) plays a key role in day-length dependent physiological processes. Long days, which are accompanied by high JH titers, induce reproduction, whereas short days result in diapause due to low JH titers (de Wilde, 1984; de Kort, 1990). One of the main factors, beside JH synthesis, influencing the JH titer is enzymatic degradation of JH by a specific esterase, which occurs mainly in the hemolymph (Kramer et al., 1977). Beetles reared under short-day conditions show reduced JH synthetic activities by the corpora allata after emergence, which results in a drop of the JH titer. In addition, high activities of JH esterase (JHE) are detected in the hemolymph, with the main increase in activity between day 4 and 8 after emergence. The highest peak of JHE activity coincides with very low JH titers in the hemolymph, which suggests that the esterase is responsible for removal of the last traces of JH from circulation and tissues in order to initiate diapause (Kramer & de Kort, 1976a; de Kort & Granger, 1996). In last instar larvae, JH has to be cleared from the hemolymph to allow metamorphosis (Hammock, 1985). In the Colorado potato beetle, JHE peaks at day 3 of the last (4th) larval instar. Hemolymph JHE was purified from that stage by nondenaturing PAGE, isoelectric focusing and SDS-PAGE successively (Vermunt et al., 1997a). The enzyme appeared to be a dimer consisting of two 57 kDa subunits, which were not linked by disulphide bridges. Two isoforms of the enzyme exist with isoelectric points of 5.5 and 5.6 (Vermunt et al., 1997a).

To make a better comparison with other esterases we intend to obtain the full amino acid sequence of JHE by molecular biological techniques. So far, this has only been achieved for *Heliothis virescens* (Hanzlik et al., 1989). Partial sequences are known from *Manduca sexta* (Venkatesh et al., 1990), *Lymantria dispar* (Valaitis, 1991) and *Trichoplusia ni* (Venkataraman et al., 1994). Here we describe the cloning and sequence analysis of a cDNA encoding the JHE subunit and the detection of its transcript in larvae and short-day beetles.

MATERIALS AND METHODS

Insects

Larvae and adults of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), were reared under two different photoregimes (short-day and long-day conditions) as described previously (Koopmanschap et al., 1992).

Isolation of JHE subunit

After prepurification of JH esterase from hemolymph of 3-day-old 4th-instar larvae by preparative nondenaturing PAGE using the Prep Cell apparatus (BioRad), fractions with JHE activity and without α-naphthyl esterase activity were pooled and concentrated as described previously (Vermunt et al., 1997a). The proteins in the JHE esterase concentrate were separated by 8.5% SDS-PAGE (Laemmli, 1970) and subsequently blotted for 3 h at 100 mA on a PVDF membrane (Immobilon P,

Millipore) using 0.01 M CAPS (3-[cyclohexylamino]-1-propane-sulphonic acid) (Sigma), pH 11.0, 10% methanol as blotting buffer. After transfer, the membrane was rinsed with distilled water and the proteins were visualized with 0.2% Ponceau S in 1% acetic acid and destained by thoroughly rinsing with distilled water. The PVDF-bound 57 kDa protein band, identified as the JHE subunit (Vermunt et al., 1997a), was excised with a razor blade.

Digestion of PVDF-bound protein
A protocol of Fernandez (1994) modified by G. Bauw (Ghent, Belgium, personal communication) was used to digest the transferred protein and to elute the resulting peptides from the blot in one step. The excised protein band on the PVDF membrane was cut in 2 mm^2 pieces and subjected to digestion in 50 µl 0.5% hydrogenated Triton X-100 (RTX-100, Sigma), 100 mM Tris-HCl, pH 8.0. For each µg of estimated PVDF-bound protein, 0.05 µg of endoproteinase Lys-C (sequencing grade, Boehringer Mannheim) was added to the digestion buffer. Digestion was carried out at 37°C for 20 h. As a control, a similar piece of membrane without protein was used. After digestion the membrane pieces were centrifuged and the eluate buffer was removed and saved. The membrane pieces were washed with 50 µl 0.5% RTX-100, 0.1% trifluoro acetic acid (TFA), 100 mM Tris.HCl, pH 8.0. The supernatant of the wash was pooled with the eluate buffer.

Peptide separation using HPLC
The eluted peptides were separated by Reversed Phase-High Pressure Liquid Chromatography (RP-HPLC), using a Techogel C4 column (4.6 mm x 25 cm, pore size 500 Å ; HPLC technology) and a linear acetonitrile / water gradient of 0-60% (v/v), with 0.1% TFA as the ion-pairing agent in both eluents. The flow-rate was 0.8 ml/min. The effluent was monitored spectrophotometrically at 220 nm. Fractions of three individual peaks were collected, concentrated with a Speed Vac concentrator (Savant) and lyophilized.

Amino acid sequence analysis
Automated Edman degradation for analysis of the amino acid sequence was carried out with 100 pmol of peptide using an automated pulse-liquid phase peptide sequencer (Applied Biosystems, model 473).

Isolation of mRNA and first strand cDNA synthesis
Messenger RNA was prepared from isolated fat bodies of 2-day-old 4th-instar Colorado potato beetle larvae using the mRNA isolation kit (Pharmacia). This time point was chosen, because JHE activity peaked at day 3 of this stage (Vermunt et al., 1997a) which will probably be preceded by a JHE mRNA peak. First strand cDNA was synthesized with the First-Strand cDNA Synthesis Kit (Pharmacia) according to the instructions of the manufacturer.

PCR with degenerate primers

For amplification of a fragment of JHE cDNA, RT-PCR was performed on mRNA isolated from 2-day-old 4th-instar larvae. Degenerate oligonucleotides were designed on basis of the amino acid sequences of the HPLC-purified peptides and synthesized by Pharmacia Biotech (The Netherlands). PCR was performed on single stranded cDNA as template, in a volume of 50 µl containing Taq buffer (Gibco BRL), 2.5 mM MgCl$_2$ and 0.2 mM dNTP s, 25 pmol of each primer and 1.25 U Taq DNA polymerase (Gibco BRL). The thermal cycle profile for PCR amplification was as follows: 5 min at 94°C; 2 cycles of 30 s at 94°C, 30 s at 37 °C, ramp of 5 s / °C,1 min at 72 °C; 30 cycles of 30 s at 94 °C, 30 s at 51 °C, 1 min at 72 °C; 5 min at 72 °C using a Thermal cycler (Hybaid). The resulting 1.3 kb PCR product was cloned into pT7Blue (Novagen).

Screening of cDNA library

A cDNA library was used, which was constructed with mRNA isolated from whole 6-day-old short-day beetles using the ZAP-cDNA synthesis kit of Stratagene (de Kort and Koopmanschap, 1994). The cDNA library was screened by hybridization with the 1.3 kb cloned PCR product. The 1.3 kb fragment was purified by electro elution (LKB) and labeled with [α-^{32}P]dATP by random prime labeling (Gibco BRL). The hybridization was carried out at 65°C and the membranes were washed under stringent conditions (last wash in 0.1 SSC, 0.1% SDS at 65°C) by standard procedures (Sambrook et al., 1989).

5'RACE

To identify the 5'-end of the gene, 5'RACE system for Rapid Amplifcation of cDNA Ends (Frohman et al., 1988) was used. Messenger RNA isolated from 2-day-old 4th-instar larvae was used as template and Superscript II (Gibco BRL) as reverse transcriptase. First strand cDNA synthesis was initiated from a gene-specific reverse primer, GSP-1: 5' TGCGGCACCTCTTCGAAAAC 3', derived from the cDNA sequence. An oligo-dC was added to the 3' end of the single stranded cDNA using terminal deoxynucleotidyl transferase (Amersham) and dCTP. PCR was performed with a nested reverse primer, GSP-2: 5' TCTGCTATATTCATGAAGTG 3', also derived from the cDNA sequence, and two forward primers: an adapter primer,
5' AAATGGATCCTTCTAGATGC 3' and an anchor primer, 5' adapter-G$_{17}$ 3'. PCR was carried out as mentioned above, except for the amount of primers, which were 10 pmol of the adapter and anchor primer and 25 pmol of GSP-2. Thermal cycle profile used for 5'RACE-PCR was as follows: one cycle of 5 min at 95 °C, 2 min at 51°C, 40 s at 72°C; 30 cycles of 40 s at 94°C, 2 min at 51°C, 3 min at 72°C; 15 min at 72°C. The resulting PCR product of 650 bp was analyzed by 1% agarose gel electrophoresis, purified by electro elution and cloned into pT7Blue (Novagen) for further analysis.

Sequence analysis

DNA sequencing was performed automatically on an Applied Biosystems, 373 DNA Sequencer-stretch, wtr 48 cm, using the kit ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit, with Amplitaq DNA Polymerase, FS. The nucleotide sequence of the cDNA and its deduced amino acid sequence were analyzed by using ESEE (Eyeball Sequence Editor) and the GCG package (Genetic Computer Group, University of Wisconsin, Madison, U.S.A.) as software.

Northern and Southern blot analysis

Northern blot hybridization was carried out on RNA samples after electrophoretic separation in formaldehyde-1.2% agarose gels and capillary transfer to Hybond N+ membranes (Amersham), as described in Sambrook et al., (1989). DNA fragments were analyzed by Southern blot analysis using standard procedures (Sambrook et al., 1989).

RESULTS

Preparation and isolation of peptides

Prepurification of JHE from hemolymph of 3-day-old 4th-instar larvae by preparative nondenaturing PAGE resulted in a clear separation of JHE activity from all α-naphthyl esterase activity and from most of the major hemolymph proteins (Vermunt et al.,1997a). Subsequently, SDS-PAGE was used to isolate the 57 kDa protein which was identified previously as the JHE subunit (Vermunt et al., 1997a).

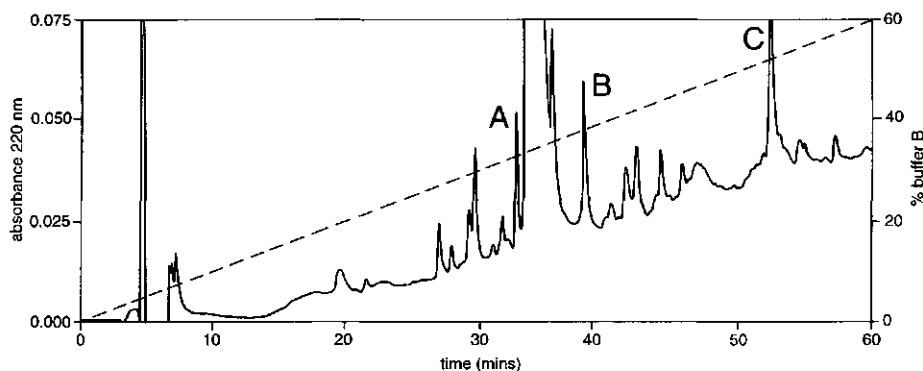Fig. 1. RP-HPLC chromatogram of endoproteinase Lys-C-digested 57 kDa protein from the Colorado potato beetle. The peptides from the peaks eluting at 34.0 min (A), 39.2 min (B) and 53.9 min (C) were sequenced. The broad peak eluting at 35 min was due to the protein staining agent Ponceau S. Eluens A: water / 0.1 % TFA; eluens B: acetonitrile / 0.1% TFA. Lines: ----, % eluens B; —— , UV absorbance at 220 nm.

After electroblotting, the amino acid sequence of the N-terminus of the protein was determined by automated Edman degradation, but the N-terminus appeared to be blocked. Because it could not be sequenced directly, the amino acid sequence of the N-terminus was determined after a mild acid deblocking (LeGendre *et al.*, 1993). The resulting chromatograms showed a considerable background probably due to hydrolysis of the protein by the acid treatment, but some amino acids were clear: T-x-K-x-x-R-V-x-x-L-x-H-V-P-.

To obtain an internal amino acid sequence, the protein band on the blot was digested with endoproteinase Lys-C and the peptides were separated by RP-HPLC. Three distinct peaks, marked A, B and C (Fig. 1), were collected and the peptides were sequenced. These peaks were absent in a HPLC run of an eluate from a digestion of a blank piece of blot. The amino acid sequences of the respective peptides are shown in Fig. 2.

The measured molecular mass of peptide A (2323 Dalton) from the HPLC run was very close to the theoretically calculated molecular mass of 2322.56 Dalton from the 22 residue sequence. The peptide sequence ended with a lysine (K), which was expected after digestion with endoproteinase Lys-C. Peptides B and C were only partially sequenced from the N-terminus, since the amino acid signals became too weak after residue 12 in case of peptide B and after residue 15 of peptide C.

```
Peptide A
 Y  G  T  A  V  Q  W  I  G  T  V  S  E  A  T  V  Q  P  G  E  T  K
TACGGIACIGCIGTICAATGGAT 3'                          Primer Est-4 (forward)
   T                  G

Peptide B
 A    D    A    R    G    I    H    I    H    F    Q    F
          5' GI  GGI ATA CAC ATA CAC TTC C 3'        Primer Est-2 (forward)
                     C   T   C   T   T
                     T       T

              3' CCA TAI GTA TAI GTA AAA GTC AA 5'   Primer Est-5 (reverse)
                     C       G       G   G   T
                     G
                     T

Peptide C
 I    P    V    V    G    E    F    L    A    P    L    I    G    F    F
          5' GTI GGI GAA TTC CTI GCA CC 3'          Primer Est-1 (forward)
                 G   T T     C
                             G
                             T
ATA CCI GTI GTI GGI GAA TT   3'                     Primer Est-3 (forward)
    C                G
    T
```

Fig. 2. N-terminal amino acid sequences of peptides from 57 kDa JHE subunit of *L. decemlineata* digested with endoproteinase Lys-C and the nucleotide sequences of the degenerate primers constructed based on the respective peptides. The forward primers were used in a PCR combined with oligo $(dT)_{18}$ as reverse primer and with primer est-5 as reverse primer. I: inosine.

```
L. decemlineata JHE:      K - Y G T A V Q W I G T V S E A T V Q P G E T K
L. decemlineata AChE:     K - P V P I D P W H G - I L D A T K Q P N S C F   54
D. melanogaster AChE:     K - P V P A E P W H G - V L D A T G L S A T C V  105
H. virescens JHE          K E L E P L E P W D N - I L N A T N E G P I C F   71
M. persicae E4:           K E P Q P V Q P W L G - V W N A T V P G S A C L   67
C. pipiens B1:            K A P V P P Q K W T E T - L D C T Q Q C E P C Y   69
```

Fig. 3. Alignment of amino acid sequence of a fragment (peptide A) of the putative *L. decemlineata* JHE with other insect esterases. Position of the last residue in the protein is as indicated (see references). Underlined amino acids and printed in bold are identical in at least four of the six aligned sequences. Amino acids printed in bold only, appear in *L. decemlineata* JHE and in one or a few other sequences. The first residue of *L. decemlineata*, K (lysine) was deduced as the peptide resulting from endoproteinase Lys-C digestion has to be preceded at the N-terminus by a lysine.
Animal species, enzyme and reference: *Leptinotarsa decemlineata* JHE (this study); *L. decemlineata* acetylcholine esterase (Zhu & Clark. 1995) *Drosophila melanogaster* acetylcholine esterase (Hall & Spierer, 1986), *Heliothis virescens* JHE (Hanzlik *et al.*, 1989), *Myzus persicae* carboxyl esterase E4 (Field *et al.*, 1993), *Culex pipiens* esterase B1 (Mouchès *et al.*, 1990).

The amino acid sequence of peptide A showed a considerable degree of similarity to peptide fragments close to the N-terminus of acetylcholine esterases from *Drosophila melanogaster* (Hall & Spierer, 1986) and *L. decemlineata* (Zhu & Clark, 1995), of JHE from *Heliothis virescens* (Hanzlik *et al.*, 1989), of carboxyl esterase E4 from *Myzus persicae* (Field *et al.*, 1993) and of esterase B1 from *Culex pipiens* (Mouchès *et al.*, 1990) (Fig. 3). This similarity to other insect esterases, in some cases 30% identity, strongly suggested that the isolated peptide A was derived from an esterase. The length of the amino acid sequences of peptide B and C was too small for proper alignment with other esterases at this point, but their position was most likely more to the C-terminus.

Cloning and sequencing of the cDNA

Based on the sequences of peptides A, B and C, five degenerate primers were made (Fig. 2). PCR on fat body single stranded cDNA of last instar larvae with different primer combinations finally resulted in a 1.3 kb product with primers est-4 and est-5. After cloning and sequencing of the amplified cDNA, the reading frame of the entire fragment was open (Figs 4 and 5). The amino acid sequence of peptide A and B were indeed found at the N- and C-terminus respectively. Peptide C was localized internally in the translated PCR product (Figs 2, 4, 5). As expected, each peptide was preceded by a lysine (K) residue.

Using this 1.3 kb PCR fragment as a probe, 200,000 clones of a cDNA library (de Kort and Koopmanschap, 1994) were screened to identify the 5' and 3' ends of the coding sequence. After primary and secondary screening, one clone was isolated from which the insert showed strong hybridization with the cloned 1.3 kb PCR fragment on a Southern blot. The size of the cDNA insert in this clone was 1.6 kb. Restriction enzyme analysis indicated overlap with the 1.3 kb PCR product (data not shown), which was confirmed by nucleotide sequence analysis of the 1.6 kb

fragment (Figs. 4, 5, sequence not in italics). The 3' end of the cDNA contained an untranslated region of about 100 nucleotides and included an AATAAA transcription termination signal.
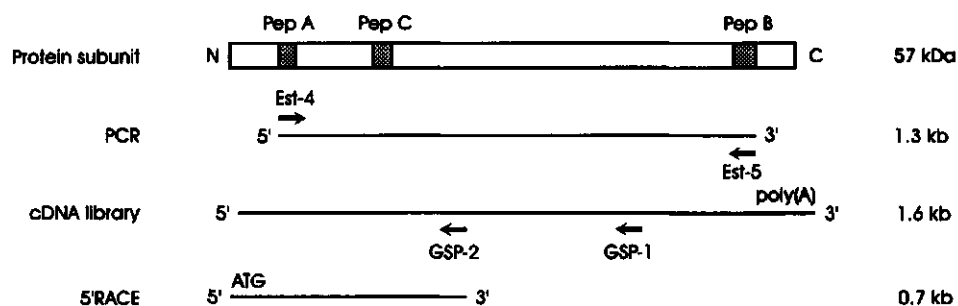
The deduced amino acid sequence from the cDNA clone did not contain the complete open reading frame of JHE because the ATG start codon was missing. To obtain the 5' end of the cDNA, 5'RACE was carried out with isolated fatbody mRNA from 2-day-old 4th-instar larvae. Two nested reverse primers (GSP-1 and GSP-2) were synthesized, based on the nucleotide sequence of the 1.6 kb cDNA. A product of 650 bp was generated and cloned in pT7Blue. After sequencing of the 5'RACE product, a potential ATG start codon was detected. The deduced amino acid sequence of this 650 bp product overlapped perfectly in 204 amino acid residues with the clone isolated from the cDNA library. Assuming that the first nucleotide triplet ATG is the start codon of translation, only 7 additional amino acids were further identified at the 5' end of the cDNA as compared to the sequence of the 1.6 kb clone of the cDNA library. The complete open reading frame of the putative JHE was determined to be 515 amino acids long (Fig. 5). No further 5' non-coding sequences were identified.

```
ATGGGTTGTTTCGAAGTTAAACCAAAGGCTAACAGAATCGTTTTGGAACACAATTCAGGC      60
 M  G  C  F  E  V  K  P  K  A  N  R  I  V  L  E  H  N  S  G        20

GTTTTTCCAGCAACAAGAAAAAATTTCCGAGTCTACAATCTTGGCCATGTACCAGTGAAA     120
 V  F  P  A  T  R  K  N  F  R  V  Y  N  L  G  H  V  P  V  K        40
       ↓
GCAGAGGCCAAATATGGAACTGCAGTGCAATGGATAGGAACGGTATCTGAAGCAACAGTT     180
 A  E  A  K  Y  G  T  A  V  Q  W  I  G  T  V  S  E  A  T  V        60

CAGCCCGGAGAAACAAAATATCTGGACATGAAGGGTAAACCGTACTACGTCACAGGAGTT     240
 Q  P  G  E  T  K  Y  L  D  M  K  G  K  P  Y  Y  V  T  G  V        80

aaactgacgaatgacaacagtaataccagagccctggttcacgttggacagcaaggccag     300
 K  L  T  N  D  N  S  N  T  R  A  L  V  H  V  G  Q  Q  G  Q       100

CGCCACTCTTGGTACATAAATAAAGAACCCGATTGGGATCTGAAGATAGACATTACTGAA     360
 R  H  S  W  Y  I  N  K  E  P  D  W  D  L  K  I  D  I  T  E       120

TTATCTAAAACACTCGTAGAATTCGGAGTCGGAAAAATACCAGTCGTAGGAGAGTTTTTA     420
 L  S  K  T  L  V  E  F  G  V  G  K  I  P  V  V  G  E  F  L       140

GCTCCCTTGATCGGATTCTTCTGGCCAGAATCTGAACCCAGCATCTGGGACCAAGTCAAA     480
 A  P  L  I  G  F  F  W  P  E  S  E  P  S  I  W  D  Q  V  K       160

GATCAAGTCGAAGAAATGATTGATACTAAAACCAATGAAGTTATCACAGGAATTCTGGGA     540
 D  Q  V  E  E  M  I  D  T  K  T  N  E  V  I  T  G  I  L  G       180

GGTGACCTCAGGCACTACAAGAAGAGGATTCAAGTGCTTGAAGAGGAATTGGACAGACAC     600
 G  D  L  R  H  Y  K  K  R  I  Q  V  L  E  E  E  L  D  R  H       200

GAGGACGTTTCTGGCCACTTCATGAATATAGCAGAGGACATGATAGGTTTCGAGCAGAAG     660
 E  D  V  S  G  H  F  M  N  I  A  E  D  M  I  G  F  E  Q  K       220

TTTATATTCAGGAAAGAGGATAACTCCAGAGCTGGTGAAATAAACTATCTGTTACTTCCC     720
 F  I  F  R  K  E  D  N  S  R  A  G  E  I  N  Y  L  L  L  P       240

ATGTTCTCTTCATTAGTATCCCTGAAAATAACTTTCCACCAATTCGGTATTCTGAACAGC     780
 M  F  S  S  L  V  S  L  K  I  T  F  H  Q  F  G  I  L  N  S       260

GAGAAGATTGGGCTGTCAGAAAAAAATGTGCAGAGGCTCAAGGACTATTCAAAGAAGCTA     840
 E  K  I  G  L  S  E  K  N  V  Q  R  L  K  D  Y  S  K  K  L       280

CTTCAAGGAACAGATGGCGCTATCGAGCACATCACGAGCGTTTTGAATGAACGCATTGAA     900
 L  Q  G  T  D  G  A  I  E  H  I  T  S  V  L  N  E  R  I  E       300

TACGAGATGAACAACTGTATTCCAGACCACATTTACGACGTCATGGTCACTGTGCGGACA     960
 Y  E  M  N  N  C  I  P  D  H  I  Y  D  V  M  V  T  V  R  T       320

TATTGTGGACTCAACGGAACTGAATACATCGCTTACTGGAATCACATCTTGGAACATCCG    1020
 Y  C  G  L  N  G  T  E  Y  I  A  Y  W  N  H  I  L  E  H  P       340

GAATCGACAACAAAACCATACAATGACGTCATAACTTACTCCACCGTGTTCGGCTCCCCA    1080
 E  S  T  T  K  P  Y  N  D  V  I  T  Y  S  T  V  F  G  S  P       360

ACTCCTATGCAAGCCAGGCAAATGGTTTTCGAAGAGGTGCCGCAGCCACTCCAACCGAAG    1140
 T  P  M  Q  A  R  Q  M  V  F  E  E  V  P  Q  P  L  Q  P  K       380

CTCGTCGACGGAAAAACGCAATAAGATTTCGGGGATCGATGTCAGCATATGGAGGTACAAC    1200
 L  V  D  G  K  R  N  K  I  S  G  I  D  V  S  I  W  R  Y  N       400

ATAAGCGGTGCCACTCCCAAAATCGGGGGTCTCATGGTCTCTTTCGAGAATGGAGATACG    1260
 I  S  G  A  T  P  K  I  G  G  L  M  V  S  F  E  N  G  D  T       420
```

```
TATAAAATGGGAGACTTTTCTGGAGAGAAACATCACGTTGATTTCAAAGAGGCTGTATGT    1320
 Y  K  M  G  D  F  S  G  E  K  H  H  V  D  F  K  E  A  V  C     440

ACAAGATTGAGTGCCTGGGGTGATGGAGATTTGGATTACATGGAGTTTGCACTTTCTGAC    1380
 T  R  L  S  A  W  G  D  G  D  L  D  Y  M  E  F  A  L  S  D     460

GGTCGCATAATGGGTTTTGGCACCAAAGCAGACGCTCGGGGTATCCACACGGACTTTCAA    1440
 G  R  I  M  G  F  G  T  K  A  D  A  R  G  I  H  T  D  F  Q     480
 ↓
CTTGAAAATCATCACATCGCTGGTATATACTTGGGCGACGACAGAGCAGGGCTAGATGGT    1500
 L  E  N  H  H  I  A  G  I  Y  L  G  D  D  R  A  G  L  D  G     500

CAAGCTGCCAATATTGCAGTGTCATATCAATTGACACCTGAAAAATGAtcaacagccagt    1560
 Q  A  A  N  I  A  V  S  Y  Q  L  T  P  E  K  *                515

cttctagtcggcccacttactgatatgttaatggcatttctcgaaaagtatttaaaattg    1620
agcttatgtaataataaatggttcataaaataaaaaaaaaaaaaaaaaa               1669
```

Fig. 5. Nucleotide sequence and deduced amino acid sequence of JHE cDNA from the Colorado potato beetle. Internal amino acid sequences derived from peptides of a proteinase Lys-C digest are underlined and printed in bold. The stop codon TGA is marked with an asterisk. The putative polyadenylation signals aataaa are underlined. The 5' and 3' end of the PCR product of 1.3 kb are indicated with arrows. The seven additional amino acids obtained by 5'RACE at the 5' end of the cDNA are printed in italics. Potential N-glycosylation sites (N-x-S or N-x-T) are double underlined.

Analysis of amino acid sequence

The entire amino acid sequence was deduced by computational translation of the nucleotide sequence of the cDNA clone and the 5'RACE product (Fig. 5). The open reading frame potentially encoded 515 amino acid residues with a predicted molecular weight of 58,212 Da. Peptides A, B and C were found in the sequence (Fig. 5, bold printed and underlined sequences). Three out of the last five residues of peptide B as determined by amino acid sequencing were not identical to the translated sequence, probably due to the low signals at the end of the sequencing of peptide B causing uncertainties.

The complete sequence did not show significant similarity to other esterases or proteins present in databases (PIR, Swiss-Prot, GenBank, EMBL) as searched with the programs BLAST, FASTA and BLOCKS. The typical motif around the active serine residue of many esterases and proteases, G-x-S-x-G (Brenner, 1988), was not present, as such, in the sequence of JHE from the Colorado potato beetle. However, other functional esterase motifs were found around the same positions as in other insect esterases (Fig. 6) and peptide A showed similarity to other insect esterases (Fig. 3). Two potential N-glycosylation sites (N-x-S or N-x-T) were found at position 325 and 400 (Fig. 5, double underlined).

RF motif
L. decemlineata JHE: P V K A E A **K Y** G T A V Q   50
H. virescens JHE: Q P V G E L **R F** K E L E P   53
L. decemlineata AChE: P P I E Q L **R F** K K P V P   57
M. persicae E4:   P P V Q N N **R F** K E P Q P   49
C. pipiens B1:   A P E G E L **R F** K A P V P   51

DQ motif
L. decemlineata JHE: S I W D Q V K **D Q** V E E M I D T K T N   172
H. virescens JHE: P G N A G L R **D Q** V T L L R W V Q R N   184
L. decemlineata AChE: P G N M G L W **D Q** I L A I R W I K D N   207
M. persicae E4:   T G N N G L K **D Q** V A A L K W I Q Q N   174
C. pipiens B1:   P G N A G L K **D Q** N L A I R W V L E N   174

Catalytic E
L. decemlineata JHE: G L N G T **E** Y I A Y W N H I L   337
H. virescens JHE: G F T S S **E** C E T F R N R L L   341
L. decemlineata AChE: G S N H D **E** G T Y F L L Y D F   362
M. persicae E4:   S I A Q D **E** G L I F S T F L G   325
C. pipiens B1:   G G T S E **E** G L L L L Q K I K   332

GxxHxxD/E motif
L. decemlineata JHE: F S **G** E K **H** H V **D** F K E A V   439
H. virescens JHE: H E **G** V G **H** I E **D** L T Y V F   454
L. decemlineata AChE: W M **G** V I **H** G D **E** V E Y V F   475
M. persicae E4:   G S S P T **H** G D **E** T S Y V L   448
C. pipiens B1:   L R **G** T A **H** A D **E** L S Y L F   450

Fig. 6. Alignment of functional motifs from L. decemlineata JHE (this paper), H. virescens JHE (Hanzlik et al. 1989), L. decemlineata acetylcholine esterase (Zhu & Clark, 1995), M. persicae E4 (Field et al., 1993), C. pipiens B1 (Mouchès et al., 1990). The given positions are from the last residue in the mature proteins. In L. decemlineata JHE in which the N-terminus was not exactly determined (see also discussion) and in C. pipiens B1, the positions given are from the complete coding regions. The functional motifs were determined through three-dimensional structure analysis in Torpedo californica acetylcholine esterase (Sussman et al., 1991), mutational study in JHE from H. virescens (Ward et al., 1992) and alignment combined with structural studies of hydrolases (Ollis et al., 1992; Cygler et al., 1993).

Size of the transcript

The size of the JHE transcript was determined on a Northern blot, containing the same amounts of total fat body RNA from different stages of the beetle, using the cloned 1,3 kb PCR product as a probe. The same transcript size of 1.7 kb was detected in larvae as well as in adults (Fig. 7). This size agreed well with the size of the insert of the cDNA clone (1.6 kb). The smear visible in the lower region of the gel might be due to mRNA degradation or non-specific hybridization.
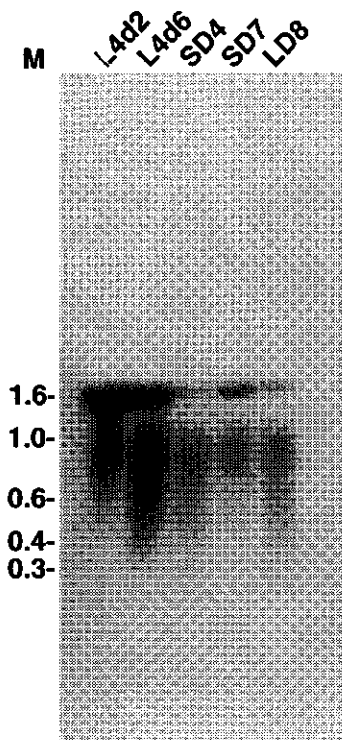
M   L4d2 L4d6 SD4 SD7 LD8

1.6-
1.0-

0.6-

0.4-
0.3-

Fig. 7. Expression of JHE transcript. Northern blot analysis of 10 µg of total RNA from fatbodies of 4th instar larvae (day 2 and 6), short-day beetles (day 4 and 7) and long-day beetles (day 8) hybridized with a $[\alpha\text{-}^{32}P]$ labeled 1.3 kb fragment of JHE cDNA. M, molecular size markers in kilobase.

If the same Northern blot was hybridized with an actin probe (Files *et al.*, 1983) as internal control for mRNA levels, the signal of larval samples was about 2-3 times stronger than that in samples from adults (data not shown). Since equal amounts of RNA were loaded per lane, this suggests that more mRNA relative to total RNA was present in larvae than in adults. Densitometric measurements of the bands revealed a four times higher level of JHE mRNA in fat bodies from 7-day-old beetles reared under short-day conditions than in 8-day-old beetles from long-days (Fig. 7; repeated with different RNA sources and normalized with actin mRNA). This indicates a higher gene expression of JHE in short-day beetles than in long-day beetles, which is in agreement with the developmental profile of JHE activity in the hemolymph of the Colorado potato beetle (Kramer & de Kort, 1976a; Vermunt *et al.*, 1997a).

DISCUSSION

The amino acid sequence presented here encodes a 57 kDa protein of the Colorado potato beetle, which was identified previously to be the subunit of the JHE dimer (Vermunt *et al.*, 1997a). Three peptides derived from an endoproteolytic digestion of the 57 kDa protein were found in the translated cDNA sequence. It is likely that the encoding protein was the major protein of the 57 kDa band because all three

peptides were localized in the same sequence. Nevertheless, the possibility still exists that other, minor, co-migrated with the 57 kDa protein band. Therefore, more evidence is required to support the identification of the sequenced cDNA as the JHE gene. This will be presented below. The possibility that the cloned cDNA codes for a general carboxyl esterase can be excluded because during the prepurification of the protein by nondenaturing PAGE all α-naphthyl esterase activity was clearly separated from the JHE activity (Vermunt et al., 1997a).

If the N-terminal sequence obtained after Edman degradation of the 57 kDa subunit was aligned with the complete amino acid sequence from the cDNA analysis of Figure 5, then the N-terminus of the mature protein starts with a threonine (T) at position 25 of the amino acid sequence. The potential signal peptide would then be 24 residues long. It possesses the properties of other signal peptides (von Heijne, 1986), like a hydrophobic core of three residues (I_{13}-V-L) surrounded by more hydrophobic residues. Proline at position -2 and phenylalanine at position -3 from the potential cleavage site, rarely occurs at these positions. If the determined N-terminus was derived from a hydrolysis product, the real N-terminus of the mature protein should be positioned more to the N-terminus of the complete sequence. According to the method of von Heijne (1986), the best possible predicted cleavage site ($\Downarrow$) should be -H-N-S-G-V-F-P_{23} $\Downarrow$ A-T-R-K-. Using the amino acid sequence observed for the mature protein, with T-R-K- at the N-terminus, the program Peptidesort (GCG) predicts a molecular weight of 55,579.6 Dalton and an isoelectric point of 5.54, which are very close to the experimentally determined values of 57 kDa and pI s of 5.5 and 5.6 (Vermunt et al., 1997a). A small shift of the position of the N-terminus of the mature protein does not much alter the calculated values.

The complete sequence of the 57 kDa protein from the Colorado potato beetle had only limited homology to JHEs and other esterases present in databases. This was probably also the explanation for unsuccessful earlier attempts to identify the cDNA for JHE of the Colorado potato beetle. Screening of a Colorado potato beetle cDNA library with JHE cDNA from H. virescens (Hanzlik et al., 1989) under low stringency conditions did not result in isolation of any positive clones. Also PCR with degenerate primers, constructed on basis of conserved regions in esterases, was unsuccessful in the amplification of a fragment of the beetle JHE (de Kort, unpublished). Purification and internal amino acid sequencing of the enzyme subunit proved to be the ultimate key to clone the cDNA.

The similarity of peptide A from a proteinase Lys-C digest of the 57 kDa protein (Figs. 3; Fig. 5, position 45-66) to other insect esterases suggests that this protein can be identified as an esterase from the Colorado potato beetle. If sequences of different esterases were compared, primarily the residues positioned at or near the catalytic site were conserved (Sussman et al., 1991; Ward et al., 1992; Cygler et al., 1993). Some motifs which appeared in other insects esterases and which were experimentally determined to be essential in enzyme activity, were also present in the 57 kDa protein from the Colorado potato beetle (Fig. 6). Four out of five motifs which play a role in the catalytic mechanism of insect esterases (Ward et al., 1992; Ollis et al., 1992; Cygler et al., 1993) were present or present in a similar context (K-Y instead of R-F in R-F motif). Although the putative JHE from L. decemlineata is

more distinct from the other esterases and alignment with complete sequences of other esterases was difficult, the motifs could be aligned around the same position of each sequence (Fig. 6). The G-x-S-x-G motif (the consensus around the active serine), which is present in most carboxyl esterases, was absent as such in the sequence of the Colorado potato beetle. The resistance of JHE activity from the Colorado potato beetle to diisopropylfluorophosphate (DFP, a known inhibitor of the active serine residue) (Kramer & de Kort, 1976b) supports the absence of an active serine, although the serine motif is present in Lepidopteran JHEs which are also resistant to DFP (Hanzlik et al., 1989; Venkataraman et al., 1994). A possible explanation for the absence of this motif might be the different quaternary structure. The active JHE from the Colorado potato beetle appears as a dimer (Vermunt et al., 1997a), whereas the other insect esterases are active as monomers or have catalytic sites on each subunit when they are multimers (acetylcholine esterases). A distinct quaternary structure makes it possible that the catalytic mechanism and the residues involved are different from the other esterases. Three-dimensional structure analysis followed by a mutational analysis can give an answer regarding the involvement of specific amino acids in the catalytic action.

In earlier studies, differences between partial amino acid sequences of JHEs from different Lepidopteran species have already been reported (Hanzlik et al., 1989; Venkatesh et al., 1990; Valaitis, 1991, 1992; Jones et al., 1994). So it is not surprising that JHE from the Colorado potato beetle differs from other JHEs. The positive correlation between the relative mRNA levels and the JHE activity in the hemolymph during development of the Colorado potato beetle under different photoregimes is an indication that we indeed cloned a JHE gene (Fig. 7; Vermunt et al., 1997a).

The results presented in this paper suggest that the JHE of the Colorado potato beetle is unique among the insect esterases. Future analysis of the enzymatic activity of this JHE will involve cloning of the JHE coding sequence in an appropriate expression system. Hammock et al. (1990) successfully expressed a functional JHE from H. virescens in the baculovirus-insect cell system. Analysis of the genomic form of the gene will provide the structure of the 5' flanking region including the promoter with regulatory elements. This will allow the study of JHE gene regulation in the various stages of Colorado potato beetle.

Genbank Accession Number
The nucleotide sequence of the cDNA encoding a putative JHE from the Colorado potato beetle is available from Genbank under accession number AF035423.

CHAPTER 4

Evidence for two juvenile hormone esterase-related genes in the Colorado potato beetle

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ABSTRACT

Juvenile hormone esterase (JHE) activity in the hemolymph of the Colorado potato beetle is necessary to initiate pupation in larvae as well as diapause in adults. The enzyme appears in the hemolymph as a dimer consisting of two 57 kDa subunits. The sequence of an encoding cDNA, JHE.A, is distinct from lepidopteran JHEs. In this study, RT-PCR using primers designed on the basis of the 5'- and 3'-ends of the coding region, revealed the existence of a related gene, JHE.B. The presence of two JHE-related genes was also shown by PCR amplification on genomic DNA from different individual beetles followed by restriction enzyme analysis. Both forms, probably paralogs, were transcribed since they could be amplified on messenger RNA from fat bodies. The size of the PCR products generated with mRNA and genomic DNA were both 1.6 kb, suggesting the absence of introns in the genomic JHE coding sequence. The sequence of a genomic clone, which encoded JHE.B, was 77% identical and 82% similar in amino acids compared to JHE.A. No introns were found in the coding sequence of these coleopteran JHE-related genes, in contrast to lepidopteran JHE genes. Southern blot analysis of digested genomic DNA confirmed the presence of two JHE-related genes.

INTRODUCTION

Juvenile hormone esterase (JHE) is the main enzyme degrading juvenile hormone (JH) in the Colorado potato beetle, *Leptinotarsa decemlineata* (Kramer *et al*, 1977). JH plays a key role in the regulation of metamorphosis and reproduction of insects (Riddiford, 1994; Wyatt & Davy, 1996; de Kort & Granger, 1996). In the Colorado potato beetle, peak activities of JHE coincide with a decline of the JH titer at critical times of the beetle's life cycle. JHE peaks appear in the hemolymph preceding pupation in larvae and the onset of diapause in adults (Kramer & de Kort, 1976a; Kramer *et al*, 1977; Vermunt *et al.*, 1997a). The enzyme was purified from the hemolymph of last-instar larvae and was present as a dimer consisting of two subunits of 57 kDa (Vermunt *et al.*, 1997a). A cDNA encoding the subunit was cloned and sequenced (Vermunt *et al.*, 1997b). The deduced amino acid sequence was distinct from lepidopteran JHEs (Hanzlik *et al.*, 1989; Venkataraman *et al.*, 1994). We therefore prefer to use the term JHE-related gene for this sequence until the function of this gene has been firmly established. Comparison of JHEs from different insect orders may provide information related to the question whether JHEs have been evolved from a common ancestor or independently from different ancestors.

The study of the genomic organization of JH-controlled genes in species of different insect orders may provide more insight in JH action. Common elements in the promoter region of JH-responsive genes for example, may indicate the existence of motifs which can bind a JH-receptor or an intermediating transcription factor (Jones, 1995). Furthermore, structure and expression studies of JH titer-regulating proteins (JH synthesis, binding and degradation) will contribute to our understanding of how the JH titer is controlled (de Kort & Granger, 1996).

The genomic structure of JHE genes was determined before in *Heliothis virescens* (Harshman *et al.*, 1994) and *Trichoplusia ni* (Venkataraman *et al.*, 1994). In these species only a single copy was found. In *H. virescens*, the JHE gene contained four introns. Here we describe the genomic organization of two JHE-related genes from the Colorado potato beetle, a coleopteran species. The existence of two related genes was established by PCR and Southern blot analysis. A genomic library was screened and the putative JHE gene was subcloned and sequenced. Sequence analysis revealed an exon/intron structure of the gene and the promoter region including the regulatory elements.

MATERIALS AND METHODS

Insects
Larvae and adults of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), were reared as described previously (Koopmanschap *et al.*, 1992).

Isolation of mRNA and first-strand cDNA synthesis
Messenger RNA was prepared from fat bodies isolated from 2-day-old 4th-instar Colorado potato beetle larvae using a mRNA isolation kit (Pharmacia). First-strand cDNA was made with the First-Strand cDNA Synthesis Kit (Pharmacia) according to the instructions of the manufacturer.

PCR amplification
PCR was performed on different types of template: first-strand cDNA, total genomic DNA, genomic and cDNA clones. Two µl of an 8-times diluted first-strand cDNA reaction mixture was used as template for PCR. Genomic DNA was extracted from individual fat bodies with 100 µl 5% Chelex-100 resin (BioRad) plus 4 µl 20 mg/ml Proteinase K (Merck) and incubated for at least 6 h at 56°C. After heating for 10 min at 95°C, 10 µl of this DNA extract was used for PCR. To amplify fragments of the cDNA clones, 5 ng of vector DNA was used. For genomic clones, 10 µl of 500 µl lambda phage suspension from a purified plaque was taken, 30 µl of water was added, boiled for 5 min and cooled on ice for 5 min.

      PCR was performed in a volume of 50 µl containing Taq buffer (Gibco BRL), 2.5 mM $MgCl_2$ and 0.2 mM dNTP's, 25 pmol of each primer and 1.25 U Taq DNA polymerase (Gibco BRL). Primers (Pharmacia) were derived from the cDNA sequence of JHE.A from the Colorado potato beetle (Vermunt *et al.*, 1997b). The sequences of the primers to amplify a fragment of 598 bp of the cDNA (nt 527-1124 of JHE.A) were: est-6, 5' TGCGGCACCTCTTCGAAAAC 3' (reverse) and est-7,
5' CAGGAATTCTGGGAGGTGAC 3' (forward). To amplify the JHE coding sequence of 1.6 kb: est-12, 5' GATCATTTTTCAGGTGTCAATTG 3' (reverse) and est-13,
5' ATGGCATCCAATCAAAGATAC 3' (forward) were used.

      The thermal cycle profile for PCR amplification was as follows: 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 53°C, 1 min at 72°C; 5 min at 72°C using a Thermal cycler (Hybaid). When other annealing temperatures were used, it is mentioned in the Results section. PCR products, undigested or digested by restriction enzymes, were analyzed by agarose gel electrophoresis.

5'RACE
To identify the 5'-end of the cDNA, the 5'RACE system for Rapid Amplifcation of cDNA Ends (Frohman *et al.*, 1988) was used. Messenger RNA isolated from 2-day-old 4th-instar larvae was used as template and Superscript II (Gibco BRL) as reverse transcriptase. First-strand cDNA synthesis was initiated from a gene-specific reverse primer, est-6 (previous paragraph), derived from the JHE.A cDNA sequence (Vermunt *et al.*, 1997b). An oligo-dC was added to the 3' end of the single-stranded cDNA using terminal deoxynucleotidyl transferase (Amersham) and dCTP. PCR was

performed with a 'nested' reverse primer, est-8 5' TCTGCTATATTCATGAAGTG 3', also derived from the cDNA sequence and two forward primers: an adapter primer, 5' AAATGGATCCTTCTAGATGC 3' and an anchor primer, 5' adapter-G_{17} 3'. PCR was carried out as mentioned above, except for the amount of primers, which were 10 pmol of the adapter and anchor primer and 25 pmol of est-8. The thermal cycle profile used for 5'RACE-PCR was as follows: one cycle of 5 min at 95°C, 2 min at 51 °C, 40 s at 72°C; 30 cycles of 40 s at 94°C, 2 min at 51°C, 3 min at 72°C; 15 min at 72°C. The resulting PCR product of 650 bp was analyzed by gel electrophoresis in 1% agarose, purified by electro elution and cloned into pT7Blue (Novagen) for further analysis.

Genomic library screening and subcloning
A genomic library of Colorado potato beetle DNA, previously constructed (Koopmanschap *et al.*, 1995), was screened with a 1.3 kb fragment of cDNA encoding JHE.A (nt 133-1442, Vermunt *et al.*, 1997b). Six genomic clones were isolated after primary and secondary screening. A 7.0 kb *EcoRV* fragment of one genomic clone was subcloned into pBlueScript KS and further analyzed by restriction enzyme and sequence analysis.

Sequence analysis
DNA sequencing was performed automatically on an Applied Biosystems apparatus, using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit, with Amplitaq DNA Polymerase (FS). The nucleotide sequence of the cDNA and its deduced amino acid sequence were analyzed with ESEE (Eyeball Sequence Editor) and the GCG package (Genetic Computer Group, University of Wisconsin, Madison, USA) as software.

Southern blot analysis
DNA fragments were analyzed by Southern blot analysis using standard procedures (Sambrook *et al.*, 1989). To determine the copy number of the JHE-related gene(s), 10 µg of genomic DNA from five beetles, extracted as described previously (de Kort & Koopmanschap, 1994), was digested with several restriction enzymes. DNA fragments were separated in 1% agarose gels and transferred to a Hybond N+ membrane (Amersham). Hybridization was carried out at 65°C with [α-^{32}P]dATP random prime labeled (Gibco BRL) cDNA fragments (Vermunt *et al.*, 1997b) under stringent conditions (last wash in 0.1 x SSC, 0.1% SDS at 65°C) according to standard procedures (Sambrook *et al.*, 1989).

RESULTS

Presence of two JHE-related genes
To obtain the 5'-end of the coding sequence of JHE, 5'RACE was performed on single-stranded cDNA derived from fat body mRNA of 2-day-old 4th-instar larvae (Vermunt *et al.*, 1997b). A product of 650 bp was obtained and cloned into pT7Blue.

48

The first sequenced clone overlapped with the cDNA clone, as described before. At the overlap the sequences were 100% identical (Vermunt *et al.*, 1997b). Further analysis (reported in this study) of other 5'RACE clones revealed a second sequence which was similar to the previous one. This was the first indication that probably two JHE-related genes exist in the Colorado potato beetle genome. The first gene was designated JHE.A and the second gene, JHE.B. More evidence was needed to establish the presence of two JHE-related genes.

RT-PCR on mRNA from fat bodies of 2-day-old 4th-instar larvae was performed to amplify the complete coding region with primers based on the 3'-end of the coding region of JHE.A cDNA (est-12) and the 5'-end of JHE.B (est-13). The resulting PCR products of 1.6 kb were cloned and analyzed. Restriction enzyme and sequence analysis showed that JHE.A and JHE.B were derived from two different messengers in the fat body. A restriction map of both genes is illustrated in Figure 1.
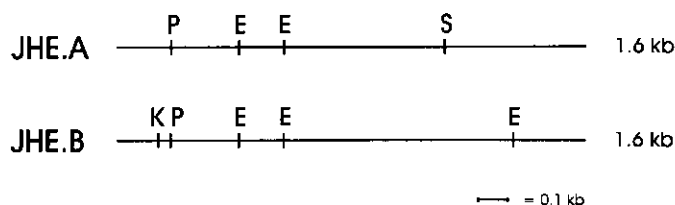
Fig. 1. Restriction enzyme map of two JHE-related genes, JHE.A and JHE.B. The maps were determined experimentally and by computer analysis of the nucleotide sequences. Restriction enzymes: E, *Eco*RI; K, *Kpn*I; P, *Pst*I; S, *Sal*I.
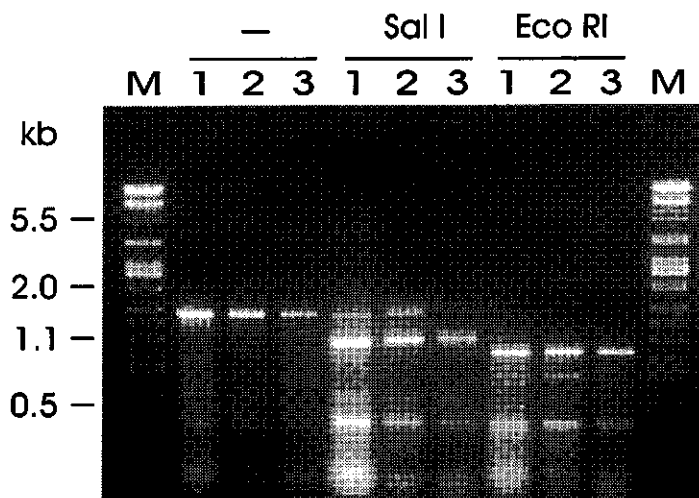
Fig. 2. Agarose gel electrophoresis of PCR products obtained by amplification of JHE-related genes using genomic DNA from individual beetles. Amplification was performed using primers designed on the 5'- and 3'-end of the coding sequence. PCR amplification was carried out at different annealing temperatures: 1, 53°C; 2, 55°C; 3, 58°C. After PCR, the products were either undigested (-) or digested by *Sal*I or *Eco*RI. M, molecular size markers.

The same primers were also used to amplify the JHE-related genes from genomic DNA of individual beetles. When an annealing temperature of 53°C was used, a 1.6 kb PCR product was seen (Fig. 2, lane 1 of left panel). The size indicated that introns do not occur in this part of the genes, because the PCR product had the same size as the one obtained by RT-PCR on mRNA. Sequence analysis of genomic clones was carried out to confirm the absence of introns (next section). Using the restriction maps (Fig. 1), the PCR products of the two genes could be distinguished by digestion with EcoRI or SalI (Fig. 2). Undigested PCR products had a size of 1.6 kb (Fig. 2, left panel). After SalI digestion, JHE.B remained undigested, whereas JHE.A gave fragments of 1.2 kb and 0.4 kb (Fig. 2, lane 1 of middle panel). EcoRI digestion resulted in detectable fragments of 1.0 and 0.4 kb with JHE.A and 0.8, 0.4 kb with JHE.B (Fig. 2, lane 1 of right panel). In each beetle the same pattern was observed, suggesting that the two genes were not allelic variations, because no homozygotes were detected. The relatively lower yield of JHE.B compared to JHE.A can be attributed to the specificity of the primers used in the PCR. If the annealing temperature was raised from 53°C to 55°C and 58°C (respectively lanes 1, 2 and 3 in Fig. 2), the unspecific products of 0.4 and 0.6 kb disappeared (Fig. 2, left panel) and amplification of JHE.B was prevented. At 58°C, only JHE.A (with SalI site) was amplified. Again, the lack of amplification of JHE.B (without SalI site, undigested band of 1.6 kb in middle panel) at this temperature was probably due to primer specificity. From these data we conclude that two JHE-related genes are present in genomic DNA of each individual beetle. The two genes can be distinguished by digestion with EcoRI or SalI.

Isolation of genomic subclone
A genomic library was screened with a 1.3 kb cDNA fragment of JHE.A (nt 133-1442, Vermunt et al. 1997b). Six genomic clones were isolated after primary and secondary screening. Four clones were selected based on the presence of PCR products after amplification with primers based on the 5'- and 3'-end of the coding sequence of JHE cDNA. The length of the PCR product was 1.6 kb and confirmed the presence of the total coding region of a JHE-related gene in these four genomic clones. This result also suggests the absence of introns, because the length of 1.6 kb was the same as after RT-PCR on mRNA. The PCR products of these four genomic clones did not have a SalI site, suggesting that all four clones contained the JHE.B gene.

Fig. 3a. Restriction enzyme map of a 7.0 kb EcoRV fragment of a genomic clone which showed a hybridizing signal with a [α-^{32}P]dATP labeled 1.3 kb fragment of the JHE.A cDNA (nt 133-1442; Vermunt et al., 1997a). The maps were determined experimentally for the complete fragment and by computer analysis for the sequenced 3.0 kb. Restriction enzymes: EI, EcoRI; EV, EcoRV; K, KpnI; P, PstI; S, SalI. The coding sequence is presented by a thick line, non-coding regions by a thin line.

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                                  AGATGATTGTGGATATTAACAAT      -961

CGAAACGTCAACTTTCATTTCATATTTTGTTCGAATTTTCAATTTTTTGCTTGAATTTTA      -901

TTTCATTGTAATTCCATTTCAGGATTTTTTGCTTAATATAAATGTTATTTCCAGCTATTC      -841

ATTTTGATTGTATTGGTCCTTTCAGGTATTTCAAATCATTTTTTATCATATTACACAATG      -781

AAAGTCCGAACCAATGAAATTCAGACAAAAAACGGCAGCAAACTAATTGAAATTGATTAT      -721

TGGTTCCAAGCCACCAAATATTTGCGATCTCTTCAAAAACCTGGGGTTATTTAAAAACTA      -661

CGAGTGGTATTTCGTTATTCATATCATTCAAAAACGAAAACTATGTTCTAAAAGTCATAT      -601

AACTGCATCAAAATAAACTGGGCATGAAACAAAAAAAATTGTGATTTTCTAATTATTGCC      -541

GCATTTCAACTAATACAATAGTGGTCCGTTTTTTAGGGAGTTGGAAATGTTCACACTGTG      -481

AAGTATGGAAATTACATTTCTTCTTACTCTTCTATACAGTGCATGAAGCAATGACTTTTA      -421

AACTCTCTAGAAAGAGAGGCAACATTCACTGCAGATGATTATGATTAGAACTTGAAAAAA      -361

ATGTACTGATAAGGCACTTTTTTTCTCATGTCAAGTGATGTAAGCTGAGAATATCTAGGA      -301

TAGAGACTTTTTCTAAAACACTTAATTCAAGAACCCCGATTCATTTTTTCTGCTGTCAAC      -241

TCATAGATAGACAAGCACTCTAGAAGTAAAATAACAAAAATAACTATGTCAAATCCTATT      -181

CCAAAACGCTCGATTGCATGTTTTTTCAACAGTTGCAGCGGTTGAATTGCACGCATGTCT      -121

GTACTAAGCTTCACATCATAAGACTATGGATAGGCGTCTGATAATTGCATGTAACTGCAA       -61

TCATTTTATTTTCTGTGATTTCAATACAAGTACGTTTTGTTTCAGGTACTAACGTGAACA       -1

ATGGCATCCAATCAAAGATACTACATGGGTGGTCACGAAGTTAAACCAAAGTCTAACAGA       60
 M   A   S   N   Q   R   Y   Y   M   G   G   H   E   V   K   P   K   S   N   R        20

TTCGCTTTGGAACACAGTTCAGGCATATTTCCATCGGTAAGAAGAAATTTCCGAATCTAC      120
 F   A   L   E   H   S   S   G   I   F   P   S   V   R   R   N   F   R   I   Y        40

AATTATGGCCGGGTACCAGTGAAAGCAGTGGCCAAATACGGAACTGCAGCGCAATGGATA      180
 N   Y   G   R   V   P   V   K   A   V   A   K   Y   G   T   A   A   Q   W   I        60

GGATCGGTAACTGGAACAACAGTCGAGCCCGGAGAAGAAAAAATTCTGGACATGCACAAC      240
 G   S   V   T   G   T   T   V   E   P   G   E   E   K   I   L   D   M   H   N        80

AGACCATACTACGTCACAGGAGTTTATCTGGAGAACGAAAACAGCAAATCCAGAGCCCTG      300
 R   P   Y   Y   V   T   G   V   Y   L   E   N   E   N   S   K   S   R   A   L       100

GTTCATGTTGGATACCAGGGCAATCGCTACTCTTGGTACACGCATAAAGAACGCGGTTGG      360
 V   H   V   G   Y   Q   G   N   R   Y   S   W   Y   T   H   K   E   R   G   W       120

GATCTGAATATAGACATTAATGAAGTAGCCAAAACACTGGTTGAATTCGGAGTCGGGAAA      420
 D   L   N   I   D   I   N   E   V   A   K   T   L   V   E   F   G   V   G   K       140

ATACCAGTCGTTGGAGAGTTTTTAGCCCCCTTGATAGGATTCTTCTGGCCAGAATCTGAA      480
 I   P   V   V   G   E   F   L   A   P   L   I   G   F   F   W   P   E   S   E       160

CCCAGCGTCTGGGACCAAGTCAAGGATCAAGTCGAAGAAATGATTGATACCAAAACCAAT      540
 P   S   V   W   D   Q   V   K   D   Q   V   E   E   M   I   D   T   K   T   N       180

GACGTTATCACAGGAATTCTGGGAGGCAACCTCAGACACCTCAAAGAAAGGATTCATGTG      600
 D   V   I   T   G   I   L   G   G   N   L   R   H   L   K   E   R   I   H   V       200

CTCAAAATGGACTTGGACAGACACAAGAACGTTTCTGGACACTTCATGAATATAGCAGAG      660
 L   K   M   D   L   D   R   H   K   N   V   S   G   H   F   M   N   I   A   E       220
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GACATGATAGGATTCGAGCATAAGTTTATGTTCAGAAATGAGGATAATGCTAGGGCTGGT    720
 D   M   I   G   F   E   H   K   F   M   F   R   N   E   D   N   A   R   A   G    240

GAAATAAACTACCTGTTACTGCCCATGTATTCTTCGTTGGTGACCCTGAGAATGACTTTA    780
 E   I   N   Y   L   L   L   P   M   Y   S   S   L   V   T   L   R   M   T   L    260

CACCAATTCGGTATTTTGAACCATGAGCAAATTGGGCTTTCAGAAGAAAATGTGCAGAGG    840
 H   Q   F   G   I   L   N   H   E   Q   I   G   L   S   E   E   N   V   Q   R    280

CTCAAGGACTACTCGAAGAGACTGATCCAAGGACCAGATGGCGCTATCAAGCACATCACT    900
 L   K   D   Y   S   K   R   L   I   Q   G   P   D   G   A   I   K   H   I   T    300

AGCGTATTGAATGAGCGCATCAATAACCAGCTGAACACATGCATTCCTGACCACGTGTAC    960
 S   V   L   N   E   R   I   N   N   Q   L   N   T   C   I   P   D   H   V   Y    320

GATGCTCTGGTCACTGTACGGACGTACTGTGGACTCAACGGAACTGAATACATCGCTTAC   1020
 D   A   L   V   T   V   R   T   Y   C   G   L   N   G   T   E   Y   I   A   Y    340

TGGAATCATCTCTTGGAACACCCGGAATCGACAACAAAGCCTTACAATGACGCTATAACT   1080
 W   N   H   L   L   E   H   P   E   S   T   T   K   P   Y   N   D   A   I   T    360

TACTCCACCATGTTCGGCTGCCCAACTCCTATCCAAGCCAGGCAAATGGTTCTCGAAGAG   1140
 Y   S   T   M   F   G   C   P   T   P   I   Q   A   R   Q   M   V   L   E   E    380

GTGCAGCAGCCACTTCAACCAAAGCTGATCAATGGAAAACGCAATAAGATTACAGGGATC   1200
 V   Q   Q   P   L   Q   P   K   L   I   N   G   K   R   N   K   I   T   G   I    400

GATGTCTGGATATGGAGGAAAAACAATAGAGGTGCCCCTCCCAAAATCGGGGGACTCAAG   1260
 D   V   W   I   W   R   K   N   N   R   G   A   P   P   K   I   G   G   L   K    420

ATCTATTTTGAAAATGGTGATTCGCATGAGTTGGGAAAATGGTCTGCTGAGAAACATTAC   1320
 I   Y   F   E   N   G   D   S   H   E   L   G   K   W   S   A   E   K   H   Y    440

GTCGAATTCAAAGGTGCTTTCTGTACAAGATTGAGTGTCTGGGGTAATGGAGCTTTGGAT   1380
 V   E   F   K   G   A   F   C   T   R   L   S   V   W   G   N   G   A   L   D    460

TACTTGGAGTTTGCACTTTCTGACGGTCGAGTTGTGGGATATGGCACCAAAAATGACGCT   1440
 Y   L   E   F   A   L   S   D   G   R   V   V   G   Y   G   T   K   N   D   A    480

AGGGGTGTCCACACAGACTTTCAACTGGAAAATCATCACATCGCTGGTATATACGTGGGC   1500
 R   G   V   H   T   D   F   Q   L   E   N   H   H   I   A   G   I   Y   V   G    500

GACGATACGGCAGGGCTAGGTGGTCAAGCTGCCAATATATCAGTGGCGTATCAATTGACA   1560
 D   D   T   A   G   L   G   G   Q   A   A   N   I   S   V   A   Y   Q   L   T    520

CCGAAGGAATGATTAGTAGCCCTTCTTCAAGTAGGCCCACTTACTGATATGTTAATGGCA   1620
 P   K   E   *                                                                  523

TTTCTCGAAAAGTATTCAAAACTAAGTTTATGTAATAATAAATGATTCATAAAATGTATC   1680

TTTTTATTCATTTCTCCCTAGTTTCAATTTGAGAAATTGTTTCAATTCAAAATACTTCTC   1740

AAAGGCAAGCTACAGATATAATGGACATATAATGAAAAACCAAACTCTGTTCAACCTATA   1800

CAGTTTCAACTGTTGCTGCATTTGATTTTGCAAATGTGCACCTCCTGCTAAGAGTAGTTT   1860

TGTATTGTTAAATTCTCCTTCAGTTAGATTTTCCAGATAGATGTTTAGAATTGAAACAAT   1920

CTTCAGTTACATACACCAGATACAAGAACACTATTTTCGAAGCATTTTTATATGTGATGC   1980

GG
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3b. Nucleotide and deduced amino acid sequence of genomic JHE.B including 5' and 3' flanking sequences. Putative ATG start codons and poly(A) signal are underlined. The stop codon is marked with an asterisk. Double-underlined sequences are putative cis-acting elements of the promoter, identified as fat body elements (Fuijwara & Yamashita, 1992).

52

To subclone the JHE.B gene, the genomic clones were digested with several restriction enzymes followed by Southern blot analysis. A 7.0 kb EcoRV fragment showed a strong hybridization signal to the 1.3 kb cDNA fragment and was cloned into pBlueScript. A restriction enzyme map of this fragment is shown in Figure 3a. The cloned fragment was partially sequenced. The sequenced part of the fragment contained a single open reading frame of 1569 nucleotides. Two possible ATG start codons were positioned close to each other (position 1 and 25, Fig. 3b). The nucleotide sequence and the deduced amino acid sequence were 100% identical to the JHE.B cDNA sequence obtained after 5'RACE or RT-PCR experiments with fat body mRNA. A comparison of the amino acid sequences of JHE.A and JHE.B is illustrated in Figure 4. The two genes showed 77% identity and 82% similarity in amino acids according to the Bestfit program from the GCG package. Indeed, no introns were found in the coding sequence as suggested before with PCR amplification.

Eleven hundred nucleotides upstream of the open reading frame were sequenced. At - 80 bp and -354 bp putative fat body elements, TGATAAA / TGATAAx (Fujiwara & Yamashita, 1992) were detected.

Copy number of JHE-related genes

To obtain the copy number of the JHE-related genes in the genome of the Colorado potato beetle, a 0.6 kb PCR product from the center of the JHE.A cDNA sequence (nt 527-1124; Vermunt et al., 1997b), was hybridized to 10 μg of genomic DNA. This DNA was digested with several restriction enzymes, which did not cut in the coding sequence of the JHE gene on basis of the sequence analysis of the cDNA. Two EcoRV fragments of approximately 5.6 and 7.0 kb in size showed a strong hybridization signal (Fig. 5). The 7.0 kb band corresponds in size to the EcoRV fragment of the genomic subclone (JHE.B; Fig. 3a). Since EcoRV does not cut in the coding sequence of either JHE.A or JHE.B, two hybridization signals can be explained either by the presence of a single copy of each of the two JHE-related genes (JHE.A and JHE.B), or by the presence of an EcoRV site within an intron of one of them. In the previous paragraph (Fig. 3b) we have ruled out the occurrence of any introns in JHE.B. In addition, after PCR amplification of cDNA and genomic DNA we obtained PCR products of the same size (Figs 1 and 2), which strongly indicated the absence of introns in JHE.A as well as in JHE.B. Thus, it seems justified to conclude that the two JHE-related genes described here occur as single copy genes in the genome of the Colorado potato beetle. These two genes are probably positioned close to each other in the genome, because after digestion with the other enzymes only one hybridization band was observed (Fig. 5). The single band after PvuII digestion was only 5.4 kb in size. Our DNA sample was poorly digested with PvuI, since a relatively strong hybridization coincided with the slot of the gel, which is mainly undigested DNA. The very faint bands observed are probably unspecific, due to the long exposure time of the film (64 h) used for this Southern blot.

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JHE.A : MGCFEVKPKANRIVLEHNSGVFPATRKNFRVYNLGHVPVKAEAKYGTAVQ : 50
JHE.B : MGGHEVKPKSNRFALEHSSGIFPSVRRNFRIYNYGRVPVKAVAKYGTAAQ : 50

JHE.A : WIGTVSEATVQPGETKYLDMKGKPYYVTGVKLTNDNSNTRALVHVGQQGQ : 100
JHE.B : WIGSVTGTTVEPGEEKILDMHNRPYYVTGVYLENENSKSRALVHVGYQGN : 100

JHE.A : RHSWYINKEPDWDLKIDITELSKTLVEFGVGKIPVVGEFLAPLIGFFWPE : 150
JHE.B : RYSWYTHKERGWDLNIDINEVAKTLVEFGVGKIPVVGEFLAPLIGFFWPE : 150

JHE.A : SEPSIWDQVKDQVEEMIDTKTNEVITGILGGDLRHYKKRIQVLEEELDRH : 200
JHE.B : SEPSVWDQVKDQVEEMIDTKTNDVITGILGGNLRHLKERIHVLKMDLDRH : 200

JHE.A : EDVSGHFMNIAEDMIGFEQKFIFRKEDNSRAGEINYLLLPMFSSLVSLKI : 250
JHE.B : KNVSGHFMNIAEDMIGFEHKFMFRNEDNARAGEINYLLLPMYSSLVTLRM : 250

JHE.A : TFHQFGILNSEKIGLSEKNVQRLKDYSKKLLQGTDGAIEHITSVLNERIE : 300
JHE.B : TLHQFGILNHEQIGLSEENVQRLKDYSKRLIQGPDGAIKHITSVLNERIN : 300

JHE.A : YEMNNCIPDHIYDVMVTVRTYCGLNGTEYIAYWNHILEHPESTTKPYNDV : 350
JHE.B : NQLNTCIPDHVYDALVTVRTYCGLNGTEYIAYWNHLLEHPESTTKPYNDA : 350

JHE.A : ITYSTVFGSPTPMQARQMVFEEVPQPLQPKLVDGKRNKISGIDVSIWRYN : 400
JHE.B : ITYSTMFGCPTPIQARQMVLEEVQQPLQPKLINGKRNKITGIDVWIWRKN : 400

JHE.A : ISGATPKIGGLMVSFENGDTYKMGDFSGEKHHVDFKEAVCTRLSAWGDGD : 450
JHE.B : NRGAPPKIGGLKIYFENGDSHELGKWSAEKEYVEFKGAFCTRLSVWGNGA : 450

JHE.A : LDYMEFALSDGRIMGFGTKADARGIHTDFQLENHHIAGIYLGDDRAGLDG : 500
JHE.B : LDYLEFALSDGRVVGYGTKNDARGVHTDFQLENHHIAGIYVGDDTAGLGG : 500

JHE.A : QAANIAVSYQLTPEK  :  515
JHE.B : QAANISVAYQLTPKE  :  515
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Fig. 4. Comparison of amino acid sequences of JHE.A and JHE.B. Identical residues in both sequences are shaded in black. Peptides obtained by proteinase Lys-C digestion of purified hemolymph JHE (Vermunt et al., 1997a) are marked with a dashed line above the sequences. All three peptides are present identically in the deduced sequence of JHE.A. One peptide (the second) appears also identically in JHE.B. The other peptides were similar in JHE.B (68% and 83% identical).
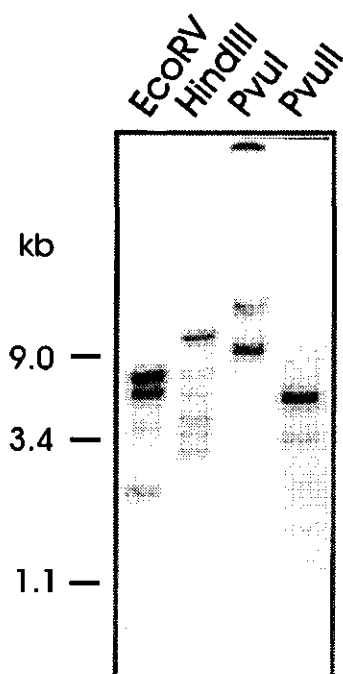
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Fig. 5. Determination of genomic copy number of JHE-related genes. Southern blot analysis of 10 µg of genomic DNA from five beetles digested with the restriction enzymes *Eco*RV, *Hind*III, *Pvu*I and *Pvu*II. Hybridization was carried out with a [α-^{32}P]dATP labeled 0.6 kb fragment from the center of the JHE.A cDNA (nt 527-1124, Vermunt *et al.*, 1997a). The DNA sample was poorly digested with *Pvu*I, since a hybridization signal coincided with undigested DNA in the slot of the gel and the vague spot around 16 kb coincided with the large smear of DNA as seen on the ethidium bromide stained gel before DNA transfer. The very faint bands in all lanes are probably unspecific, due to the long exposure time of the film (64 h) used for this Southern blot.

DISCUSSION

After characterization of cDNA encoding a putative JHE from the Colorado potato beetle (JHE.A; Vermunt *et al.*, 1997b), we now identified a second JHE-related gene (JHE.B). Both genes are transcribed, because 5'RACE and RT-PCR on mRNA from 2-day-old 4th-instar larvae yielded sequences of both genes.

JHE.A is present in the hemolymph as previously shown (Vermunt *et al.*, 1997a). It seems unlikely that JHE.B also occurs in the hemolymph. Three HPLC-separated peptides derived by endoproteinase Lys-C treatment of purified hemolymph JHE, were encoded by the cloned cDNA of JHE.A. One of the three purified Lys-C peptides was also encoded by JHE.B. However, the other two peptides showed high similarity but were not identical in JHE.B (Fig. 4). In addition, the program Peptidesort of GCG predicted an isoelectric point of 6.9 for JHE.B.

Taking into account a similar signal peptide as in JHE.A (Vermunt *et al.*, 1997b), the N-terminus of JHE.B starts at residue 25 (V-R-R-; Fig. 3b). For JHE.A, a pI of 5.5 was predicted which was the same as the experimentally determined values of 5.5 and 5.6 for hemolymph JHE (Vermunt *et al.*, 1997a). This makes it unlikely that JHE.B plays a role in hemolymph JHE activity, unless JHE.B is extensively modified after translation. It is possible however, that JHE.B plays a role within the fat body cells, which are known targets for JH (Jones *et al.*, 1994; Riddiford, 1994; Wyatt & Davy, 1996). When the JH titer drops at certain physiological stages, JH should be purged from target tissues and circulation. This may occur by expression of two JHE genes, resulting in elevated JH degradation. In future experiments, age-

55

and tissue-dependent expression studies will be carried out to detect expression of the two JHE genes in various tissues.

Hemolymph JHE from the Colorado potato beetle is only active as a dimer (Vermunt et al., 1997a). If JHE.B is not present in the hemolymph, then consequently hemolymph JHE should be a homodimer consisting of two JHE.A subunits. It is possible that the catalytic site is formed by residues of both subunits, like in some aspartyl proteases (HIV protease and pepsin; Stryer, 1995). If this is the case, the catalytic residues of both subunits should be pointed to the surface of the respective subunits instead of laying in a gorge, like in most esterases (Sussman et al., 1991; Cygler et al., 1993). This can also be an explanation for the absence of the typical esterase motif G-x-S-x-G (consensus around the catalytic serine) in both JHE-related genes (Fig. 4; Vermunt et al., 1997b). Most serine proteases and esterases are known to be inhibited by diisopropylfluorophosphate (DFP), but JHE activity from the Colorado potato beetle was shown to be resistant to this inhibitor (Kramer & de Kort, 1976b) like most JHEs (Hammock, 1985). In contrast to lepidopteran JHEs, JHE in the Colorado potato beetle is sensitive for Triton X-100 inhibition (Kramer et al., 1977; Hammock, 1985). The different quaternary structure, a dimer instead of a monomer, and its different inhibition characteristics make it possible that the catalytic mechanism for JH ester hydrolysis and the involved amino acid residues are distinct from other insect esterases. The subunits of the dimer are not linked by disulphide bridges (Vermunt et al., 1997a). If the subunits are held together by hydrophobic interactions, the inhibition by Triton X-100 might be due to breaking the JHE dimer.

The 82% similarity between JHE.A and JHE.B (Fig. 4) suggests that they are paralogs, derived from a duplication event. In *T. ni*, one and possibly two additional genes were found to be related to the JHE gene (Jones et al., 1994). Duplication of esterase genes is an established phenomenon in insects. For example, amplification of esterases is a known mechanism for resistance against organophosphorus insecticides in *Culex* (Mouchès et al., 1990) and *Myzus* (Field et al., 1993). In *Drosophila melanogaster*, a family of esterase genes is located on the genome in a cluster. Eleven putative esterase genes have been mapped within 65 kb of genomic DNA (Russell et al., 1996). Maybe the two JHE-related genes of the Colorado potato beetle also lie in a cluster of esterase genes. An indication for this is that both genes were mapped on a restriction fragment of only 5.4 kb (Fig. 5).

The data assembled on JHE and its genes thus far indicate that the JHE-related genes in the Colorado potato beetle are distinct from other characterized insect esterases. Also the absence of introns in the genes was surprising and adds further credence to this hypothesis. In contrast, the *H. virescens* JHE gene contained four introns (Harshman et al., 1994). In most eukaryotic protein-coding genes, introns are present; however in some genes, the introns were excised during evolution, like in globin genes. In this gene family, the gnat globin gene contains no introns, whereas the ancestral globin gene contained three introns (Li & Grauer, 1991). More data about JHE genes from different insect orders are needed to gain further insight in the structure, regulation and evolution of JHE genes.

Accession numbers sequences

The nucleotide sequences of the two JHE-related genes from the Colorado potato beetle are available from Genbank, the cDNA encoding JHE.A under accession number AF035423 and the genomic DNA encoding JHE.B under AF039135.

CHAPTER 5

Expression of the juvenile hormone esterase gene in the Colorado potato beetle, *Leptinotarsa decemlineata*: photoperiodic and juvenile hormone analog response.

A.M.W. Vermunt, A.B. Koopmanschap, J.M. Vlak and C.A.D. de Kort

ABSTRACT

Metamorphosis and reproduction in insects are controlled by juvenile hormone (JH). One of the factors, which regulate the JH titer in the hemolymph, is the activity of juvenile hormone esterase (JHE). JHE from the Colorado potato beetle, *Leptinotarsa decemlineata*, consists of two 57 kDa subunits. In this study, the JHE-cDNA was used as a probe to examine where and when the gene is transcribed as well as how gene expression responds to photoperiodic treatment and to topical application with a JH analog, pyriproxyfen. JHE transcripts were almost exclusively found in RNA extracts from fat body tissue in both larvae and adults. JHE-mRNA levels in the fat body correlated positively with levels of JHE activity in the hemolymph. In the last larval instar, high levels of JHE-mRNA were found in the feeding stage. In adults, reared under short-day conditions, JHE-mRNA levels were high between day 2 and day 9, which correlated with high JHE activity in the hemolymph. During these conditions, the JH titer decreases in preparation for pupation and diapause, respectively. The JHE-mRNA levels and JHE activity in the hemolymph were higher in short-day than in reproductive long-day adults. If the JH analog pyriproxyfen was applied to animals of the last larval instar on day 0 or day 3, JHE gene expression was enhanced. In contrast, if pyriproxyfen was applied to short-day adults on day 1 or day 4, the mRNA levels and the JHE activity in the hemolymph were suppressed to levels similar to those found in long-day adults. Thus, transcription of JHE is dependent on developmental stage, tissue, photoperiod and the level of its substrate JH.

INTRODUCTION

Juvenile hormone (JH) regulates many physiological processes in insects. Larvae require JH to maintain the larval state, but in the last larval instar absence of JH is necessary for the onset of metamorphosis (Riddiford, 1994). In adults, the presence of JH is a prerequisite for reproduction (Wyatt & Davey, 1996; de Kort & Granger, 1996). Therefore, regulation of the JH titer is crucial for the orderly development and reproduction of insects.

Diapause in the Colorado potato beetle, *Leptinotarsa decemlineata*, occurs in the adult stage and is characterized as a non-reproductive developmental program (de Kort, 1990). It is induced by photoperiod (de Wilde *et al.*, 1959). Under short-day conditions (10 h light: 14 h dark), beetles enter diapause 11-12 days after adult emergence by digging into the soil (de Kort *et al.*, 1982). The photoperiodic induction of diapause is mediated by a decrease in JH titer (de Kort, 1990). This decrease is the result of low rates of JH synthesis by the corpora allata and of a rise of juvenile hormone esterase (JHE) activity in the hemolymph (Kramer *et al*, 1977; Kramer, 1978a, b; Khan *et al.*, 1982a, b).

JHE from the hemolymph of the Colorado potato beetle has been purified and characterized. The enzyme consists of two subunits, 57 kDa in size, and appears in two isoforms with isoelectric points of 5.5 and 5.6 (Vermunt *et al.*, 1997a). The putative gene for this JHE has been cloned and sequenced. The size of the transcript in the last larval instar and adults is 1.7 kb. The amino acid sequence was compared to that of other insect esterases and limited homology was found around the position of some functional motifs of the JHE enzyme (Vermunt *et al.*, 1997b).

Expression of the JHE gene has been studied in *Heliothis virescens* (Wroblewski *et al.*, 1990) and *Trichoplusia ni* (Venkataraman *et al.*, 1994). In the last larval instar, high levels of JHE-mRNA were observed, which could be enhanced by JH treatment. In *T. ni*, JH induction of the prepupal JHE peak was detected within three hours after treatment.

In the Colorado potato beetle, JHE activity in the hemolymph can be enhanced by JH application to 2-4 weeks old diapausing beetles (Kramer, 1978b). In diapause JH is absent. JH application in this case results in a negative feedback mechanism, manifested by an induction of JHE and subsequently to enhanced JH degradation. On the other hand, JH application to short-day beetles during the first 3 days of adult life suppressed the JHE activity peak, which normally occurs in untreated animals on day 8 (Kramer, 1978b). These pre-diapausing beetles are not yet committed for diapause and JH application can reverse the short-day response into a long-day (reproductive) reaction. This reversal of a short-day into a reproductive response becomes particularly obvious after application of the potent JH analog pyriproxyfen (Koopmanschap *et al.*, 1989).

The expression of genes for two major hemolymph proteins has recently been described (de Kort et al., 1997). Diapause protein 1 (Dp-1) is abundantly expressed in short-day beetles, but not in long-day beetles. On the other hand, vitellogenin (Vg), which is a major protein in the hemolymph of long-day females, is not expressed in short-day females. Application of pyriproxyfen to short-day females prevented the synthesis of Dp-1 and induced the appearance of Vg (de Kort et al., 1997).

Here, we present a study of JHE gene expression in larvae, pupae and adults of the Colorado potato beetle in relation to photoperiodic treatment and JH analog application. This was carried out by correlating JHE activities in the hemolymph and the intensity of slot-blot hybridization signals with total RNA samples of fat bodies isolated from the same animals. Fat bodies were dissected, because they are considered to be the principal site of JHE synthesis (Roe & Venkatesh, 1990). The beetles were reared under two different photoperiodic regimens, short-day and long-day conditions. The question in which tissues the gene is expressed was addressed by RNA extraction from different tissues, followed by slot-blot hybridization with the JHE-cDNA probe.

MATERIALS AND METHODS

Insects
Larvae and adults of the Colorado potato beetle, Leptinotarsa decemlineata (Say), were reared on fresh potato foliage as described previously (de Kort et al., 1997). Briefly, animals were reared under two different photoperiodic regimens: long-day conditions, 16 h light : 8 h dark at 25°C and short-day conditions, 10 h light : 14 h dark, at 23°C.

JH analog treatment and hemolymph collection
Animals were treated with a single dose of 1 µg JH analog in 1 µl acetone. The analog was pyriproxyfen, 2-[1-methyl-2-(4-phenoxyphenoxy)-ethoxy]pyridine
(10% emulsifiable concentrate, Sumitomo Chemical Co. Ltd, Osaka, Japan). Controls were treated with 1 µl of acetone containing the emulsifiable solution without pyriproxyfen. The acetone solutions were applied topically as described previously (de Kort et al., 1997). Hemolymph was collected and pooled (de Kort et al., 1997) from at least five beetles for each time point. JHE activity was measured in duplicate (two different hemolymph dilutions) for each pooled sample.

JHE assay
JHE activity was measured according to a partition assay (Hammock & Sparks, 1977), as modified by Lefevere (1989), in 50 mM Tris-HCl, pH 8.0 as assay buffer. Racemic [^{3}H]-JH III (Amersham) mixed with unlabeled JH III (Calbiochem) at a final concentration of 5 x 10^{-6} M was used as substrate. Undegraded JH was extracted with iso-octane. The aqueous phase containing JH-acid was counted for radioactivity in a liquid scintillation counter (Beckman LS 6000TA) and the amount of hydrolyzed JH III was calculated.

Tissue and RNA isolation
From the same animals used for hemolymph collection, fat bodies were dissected for extraction of total RNA. Tissue dissection occurred using a microscope after submergence of the animals in Ringer solution (Khan et al., 1982a). Tissues from 5-10 animals were pooled in TRIzol reagent (Gibco BRL) for total RNA extraction according to the instructions of the manufacturer. RNA was precipitated from the aqueous phase with isopropanol, rinsed with 75% ethanol and dissolved in water. The RNA concentration was determined with a spectrophotometer (Gene-Quant, Pharmacia) at 260 nm. Other tissues used for RNA extraction such as gut epithelium, brains, ovaries, testes and flight muscles were similarly treated. In a few experiments, tissues were dissected, washed extensively with Ringer solution and collected in 50 mM Tris-HCl, pH 8.0 buffer, for ultrasonic homogenization and determination of JHE activity.

RNA analysis
To compare JHE-mRNA levels, slot-blot hybridization was carried out. Samples of 5 μg total RNA were applied to Hybond N+ membranes (Amersham) using a Bio-dotSF microfiltration apparatus (BioRad). Hybridization was carried out at 65°C with [α-^{32}P]dATP random prime labeled (Gibco BRL) 1.3 kb JHE-cDNA fragment (Vermunt et al., 1997b) under stringent conditions (last wash in 0.1 SSC, 0.1% SDS at 65°C) according to standard procedures (Sambrook et al., 1989). Previous Northern blot analysis showed that, with the above mentioned 1.3 kb JHE-cDNA fragment as probe, a single transcript of 1.7 kb was specifically detected (Vermunt et al., 1997b). This justifies the use of slot-blot hybridization for semi-quantitative analysis for JHE expression. After hybridization with the JHE-cDNA, the membrane was deprobed and hybridized with an actin probe (a gift of Dr. M.H. Roos, ID-DLO, Lelystad, The Netherlands; Files et al., 1983) as internal standard for loading equal amounts of RNA. Actin is constitutively expressed in most cells and at all developmental stages.

RESULTS

JHE gene expression in various tissues
Total RNA was extracted from different tissues, which were isolated from 2-day-old 4th-instar larvae (L4d2) and 4-day-old short-day adults (SD4), two states with high JHE activities in the hemolymph (Vermunt *et al.*, 1997a). Fat bodies, gut epithelium and brains were dissected from larvae and adults; ovaries, testes and flight muscles only from adults. In larvae as well as in short-day adults, a high level of JHE-mRNA was found in samples from fat bodies (Fig. 1). A low level was found in the testes of male adults. Brains from 4th-instar larvae showed a significant hybridization signal, which was not detected with brains from short-day adults (Fig. 1). The larval brain is very small and consequently 15 brains were necessary to obtain 5.0 µg of RNA. From one larval fat body, around 100 µg RNA was isolated. Thus, approximately 300 times more JHE-RNA was present in one fat body than in one brain from 4th-instar larvae. The hybridization of brain RNA samples could not be correlated with detectable JHE activity in homogenates of these brains. For our studies of developmental expression of JHE, we therefore decided to extract total RNA from fat bodies only.

JHE expression in fourth-instar larvae
In 4th-instar larvae reared under two photoperiodic regimens, JHE activity in the hemolymph was monitored at different times after larval ecdysis (Fig. 2a). Under long-day conditions, peaks of JHE activity were observed on day 3 and day 9. Day 3 larvae still fed intensively until they stopped on day 6 and started to dig into the soil, in preparation for pupation, which usually occurs on day 9. These data resembles data from a previous study (Vermunt *et al.*, 1997a). Under short-day conditions the peaks were lower, wider and delayed for a few days as compared to long-days. Digging was also delayed for 2-3 days. This delay is probably due to the lower rearing temperature used under short-day conditions.
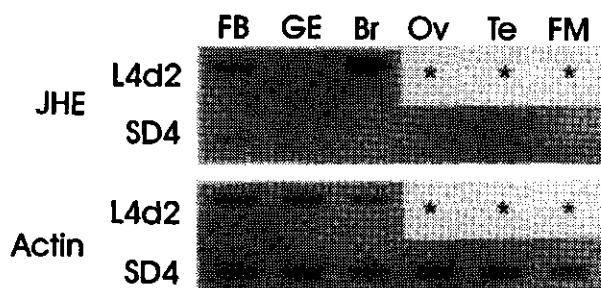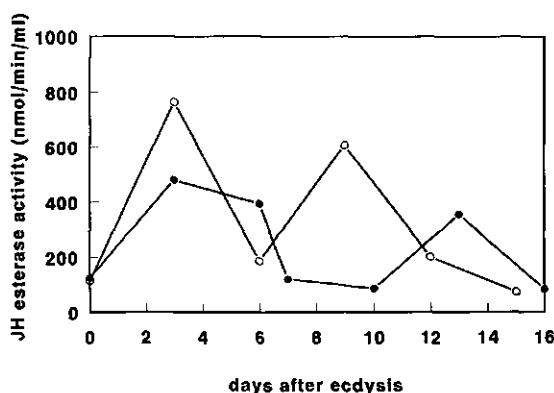

Figure 1. Slot-blot hybridization of a ^{32}P-labeled JHE-cDNA probe to 5 µg of total RNA extracted from various tissues of 2-day-old 4th-instar larvae (L4d2) and of 4-day-old short-day adults (SD4) of the Colorado potato beetle. FB, fat body; GE, gut epithelium; Br, brains; Ov, ovaries; Te, testes; FM, flight muscles. As internal standard for loading equal amounts of RNA, the same membrane was hybridized with an actin probe. In a replicated experiment, the same pattern was observed. Asterisks indicate tissues that are not developed in larvae.

The JHE-mRNA levels in the fat body were measured with slot-blot hybridization of RNA extracted at various times after molting into the fourth instar. Messenger RNA levels were high from day 2 till day 6 with a peak on day 4 (Fig. 2b). After digging into the soil, the level of the JHE-mRNA in the fat body decreased rapidly. The pattern of JHE expression observed in larvae reared under short-day and long-day conditions appeared to be very similar (Fig. 2b) which is in agreement with earlier observations that the photoperiodic response only becomes obvious in the adult stage (de Wilde *et al.*, 1959; de Kort, 1990).
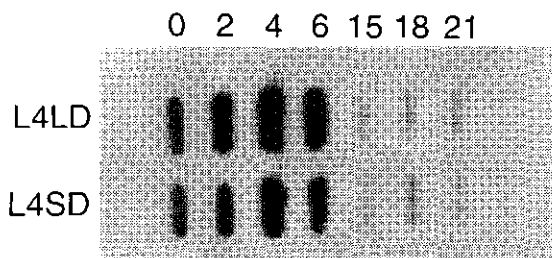
A

B

Fig. 2 **(A)** JHE activity in the hemolymph throughout the 4th-larval instar and pupal stage, reared under long-day (o) and short-day conditions (•). Data represent the JHE activity in pooled hemolymph samples of 5-6 individuals. On day 6, long-day larvae dig into the soil and on day 9 they pupate. Short-day larvae dig and pupate 2-3 days later.
(B) Slot-blot hybridization of a ^{32}P-labeled JHE-cDNA probe to 5 µg of total RNA extracted from fat bodies of 4th-instar larvae and pupae of the Colorado potato beetle at different times after the larval molt. The age of the larvae, in days after molting, is indicated above. LD, long-day conditions; SD, short-day conditions.

JHE expression in short-day and long-day adults

Hemolymph from adults reared under short-day conditions showed much higher JHE activity than hemolymph from reproductive long-day adults (Fig. 3a), with a peak on day 9. A similar peak was previously reported on day 8 (Kramer & de Kort, 1976), but the rearing temperature was 25°C at that time. From the same animals used for determination of JHE activity, fat body RNA was isolated and analyzed for JHE-mRNA levels by slot-blot experiments. In agreement with hemolymph JHE activity, JHE-mRNA levels were higher in short-day adults, between day 2 and day 12, than in long-day adults (Fig. 3b). After 11-12 days under short-day conditions, beetles burrowed into the soil in preparation for diapause.
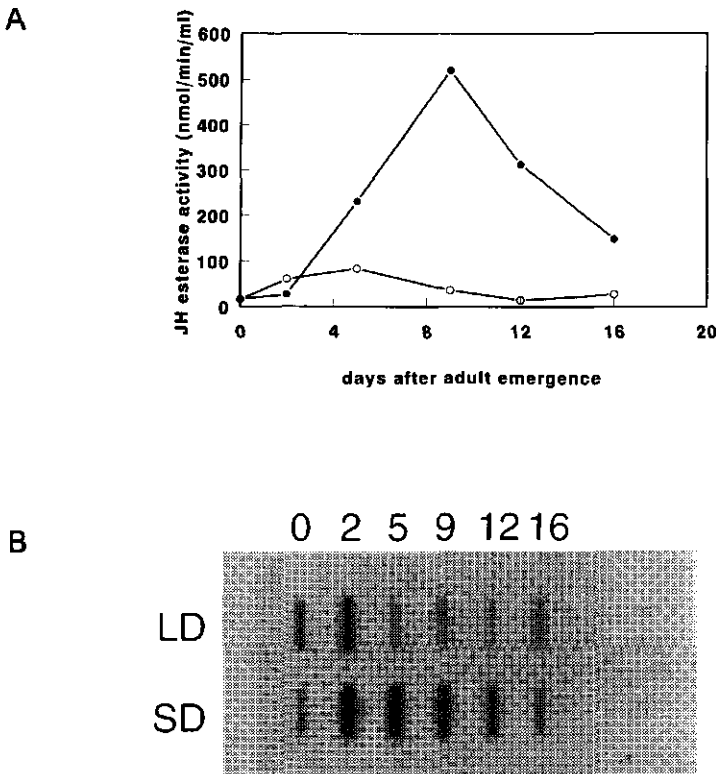
A

B

Fig. 3 (A) JHE activity in the hemolymph throughout the adult stage reared under long-day (○) and short-day conditions (●). Data represent the JHE activity in pooled hemolymph samples of 5-6 individuals.
(B) Slot-blot hybridization of a ^{32}P-labeled JHE-cDNA probe to 5 µg of total RNA extracted from fat bodies of adults of the Colorado potato beetle at different times. The age of the animals, in days after adult emergence, is indicated above. LD, long-day conditions; SD, short-day conditions.

JH analog application to fourth-instar larvae

Fourth-instar larvae reared under long-day conditions were treated with 1 µg of pyriproxyfen on day 0 and day 3. When applied on day 0, JHE activity increased with a factor 4-5 and remained high till day 9 (Fig. 4a, top panel). The JH analog treated larvae did not show digging behavior on day 6, nor later. Controls treated without pyriproxyfen but with acetone showed a similar JHE activity profile as in Figure 2a. The peaks appeared somewhat lower and wider compared with untreated long-day larvae (Fig. 2a). Control (acetone) treatment may have led to a stress reaction. Environmental stress factors, like chilling and injury, were previously reported to inhibit JHE synthesis and to cause a delay in metamorphosis (Roe and Venkatesh, 1990; Hirashima et al., 1995).

Pyriproxyfen treatment on day 3 also resulted in elevated JHE activity (Fig. 4a, lower panel). The larvae showed continued feeding and only 25% burrowed into the soil after a delay of three days. Continued feeding resulted in heavier larvae, which contained much more hemolymph than the controls.

Larvae treated on day 6 showed normal digging behavior and JHE activity in the hemolymph was not significantly affected either (data not shown). This confirms our previous observation that pyriproxyfen hardly affects gene expression when applied on day 6 of the last larval instar (de Kort et al., 1997)

The JHE-mRNA levels after JH analog treatment on day 0 and 3 are illustrated in Figure 4b. After treatment on day 0 or day 3, the JHE-mRNA levels in the fat body showed a higher hybridization signal than the controls, after an initial drop immediately after application (Fig. 4b).

JH analog application to short-day adults

Pyriproxyfen treatment suppressed both JHE activity and JHE-mRNA levels when applied to short-day animals on day 1 and day 4 (Fig. 5a, top and lower panel). The suppression was stronger when the analog was applied on day 1. JH analog application on day 8 did not significantly affect JHE activity or JHE-mRNA levels (data not shown), which confirms previous observations that pyriproxyfen is not very effective when applied to 8-day-old short-day adults (de Kort et al., 1997). The suppression of JHE activity and JHE-mRNA levels did not occur irreversibly. After a temporary drop, both the enzyme and mRNA levels started to increase again (8-15 days after each treatment, Fig. 5), probably due to endogenous degradation of the JH analog. Control females kept under short-day conditions never showed mating behavior or egg development. However, pyriproxyfen treatment on day 1 led to mating on day 5 and some oviposition on day 8. When a five times higher dose of pyriproxyfen was applied (data not shown), mating and oviposition were more pronounced. When pyriproxyfen was applied on day 4, the reversal to reproduction was less obvious.

A
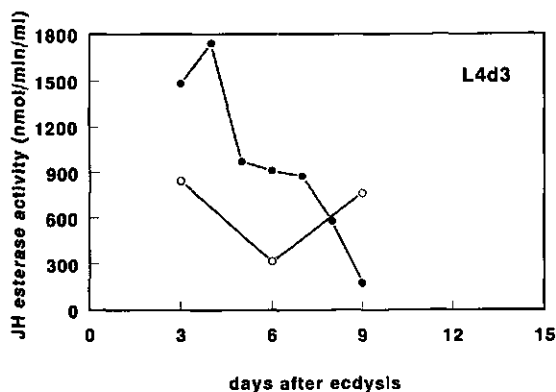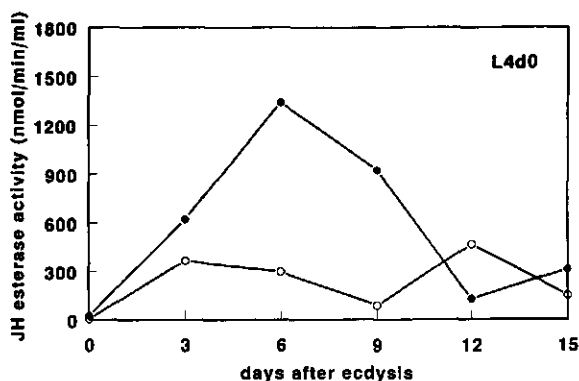
B
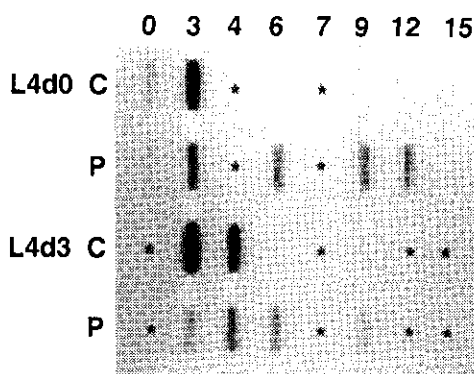
Fig. 4 (A) JHE activity in the hemolymph throughout 4th-instar larvae reared under long-day conditions after pyriproxyfen treatment on day 0 (L4d0) and day 3 (L4d3). ○, control (acetone plus emulsion solution) application; ●, pyriproxyfen (1 μg in acetone) application.

(B). Slot-blot hybridization of a ^{32}P-labeled JHE-cDNA probe to 5 μg of total RNA extracted from fat bodies of long-day 4th-instar larvae of the Colorado potato beetle at different times after pyriproxyfen treatment on day 0 (L4d0) and day 3 (L4d3) of this instar. The age of the animals, in days after the larval molt, is indicated above. C, control (acetone plus emulsion solution) application; P, pyriproxyfen (1 μg in acetone) application. At the time points with an asterisk, no RNA was applied.

67

A
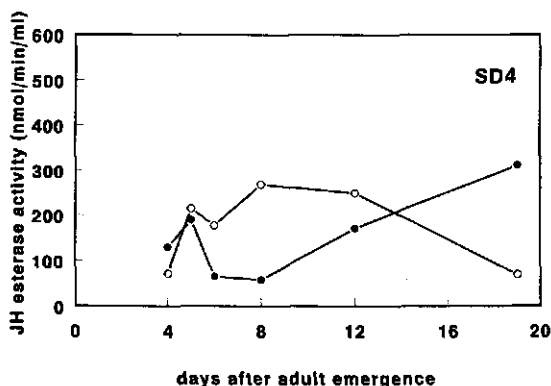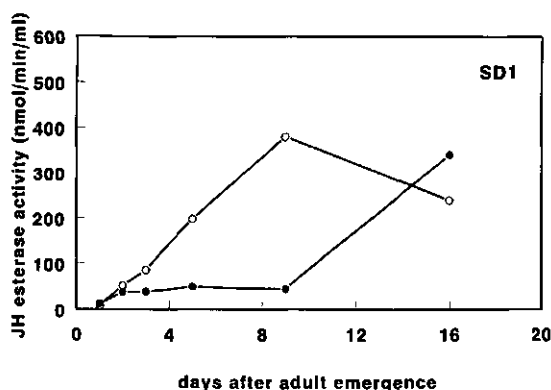
B
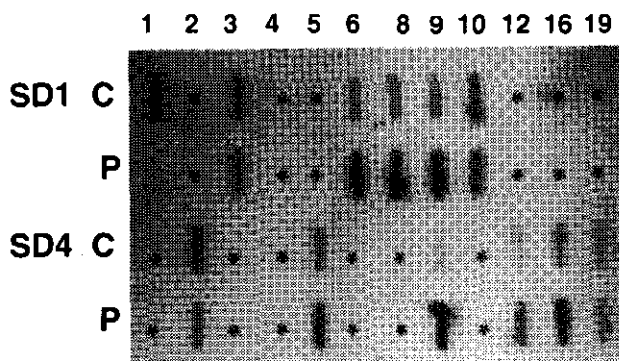
Fig. 5 (A) JHE activity in the hemolymph throughout short-day adults after pyriproxyfen treatment on day 1 (SD1) and day 4 (SD4). ○, control (acetone plus emulsion solution) application; ●, pyriproxyfen (1 μg in acetone) application.

(B) Slot-blot hybridization of a ^{32}P-labeled JHE-cDNA probe to 5 μg of total RNA extracted from fat bodies of short-day adults of the Colorado potato beetle at different times after pyriproxyfen treatment on day 1 (SD1) and day 4 (SD4). The age of the animals, in days after adult emergence, is indicated above. C, control (acetone plus emulsion solution) application; P, pyriproxyfen (1 μg in acetone) application. At the time points with an asterisk, no RNA was applied.

DISCUSSION

In this paper, we studied the expression of the JHE gene in larvae and adults, during long-day and short-day conditions and investigated the relationship between hemolymph JHE activity and fat body JHE-mRNA levels. The same hemolymph and fat body samples used in the study of expression of the gene for Dp-1 and Vg (de Kort et al., 1997) were employed for determination of JHE activity and JHE-mRNA analysis. This makes integration of data more reliable.

In contrast to Dp-1 and Vg, JHE is a minor protein in the hemolymph. This was reflected by the higher amount of total RNA necessary for detection of the JHE-mRNA. For Dp-1 only 0.5 µg of total tissue RNA was needed (de Kort et al., 1997), whereas detection of JHE-mRNA required at least 5.0 µg of total tissue RNA and an approximately two times longer exposure time of the film to the hybridized blot. Using this amount of RNA, we showed that the fat body is the major source for the production of hemolymph JHE (Fig. 1), which confirms findings reported for other species (Roe and Venkatesh, 1990). Our experiments also revealed a significant hybridization signal with RNA samples from larval brains (Fig. 1). Synthesis of JHE outside the fat body has been reported before in larvae of Manduca sexta, in which JHE activity was also detected in the brain (Jesudason et al., 1992). Brain JHE probably regulates the JH titer near its site of synthesis, the corpora allata. If brains are a source of hemolymph JHE in the Colorado potato beetle, this only takes place in the larval stage, since no detectable hybridization was observed with brains from adults (Fig.1).

The JHE gene is expressed in the last larval instar during the period of intensive feeding, but the expression rapidly decreases after the onset of digging behavior, which marks the preparation for pupation (Fig. 2b). In this connection it is worthwhile to mention that JHE activity in hemolymph from earlier instars is extremely low (A.M.W. Vermunt, unpublished results), suggesting that JHE is preferentially expressed in the last larval instar. A positive correlation was found between JHE activity in the hemolymph and the hybridization signal with larval fat body RNA (Fig 2), which suggests that regulation of the gene occurs at the level of transcription. Photoperiod did not affect JHE gene transcription in larvae, which is not surprising since the adult is the responsive stage for photoperiodic induction (de Kort, 1990). However, the second JHE activity peak observed around pupation did not correlate with an increase of JHE-mRNA in the fat body (Fig. 2). This peak of JHE activity can either be explained by extra release of JHE from the fat body due to lysis of this tissue (de Loof, 1972; Labour, 1974) or by JHE release from another source, for example the larval brain (Fig. 1; Jesudason et al., 1992).

In adults, the gene for JHE is expressed under long-day as well as short-day conditions, but at different rates. Under long-days, the activity of JHE in the hemolymph is significantly lower than in short-day beetles (Fig. 3a; Kramer and de Kort, 1976; Vermunt et al., 1997a). This correlated well with the JHE-mRNA profiles in fat body from beetles reared under the two photoperiodic regimens (Fig. 3b). Short-day conditions require higher levels of JHE expression to remove JH from the hemolymph in preparation for diapause.

The response of the JHE gene to application of the JH analog pyriproxyfen differs from that described for Dp-1 and Vg (de Kort *et al.*, 1997). If the JH analog was applied to 4th-instar larvae in the feeding stage, the level of JHE activity in the hemolymph increased. Induction of extra JHE by the JH analog suggests that larvae are already committed for metamorphosis at this stage. The high hybridization signal on day 0 also indicates that 4th-instar larvae are rapidly committed for metamorphosis. On the other hand, the change of commitment from larvae to pupae is not yet complete, since the Dp-1 gene becomes activated from day 2 (de Kort & Koopmanschap, 1994).

The first physiological difference between long-day and short-day females after adult emergence appears to be the JH synthetic activity of the corpora allata. This activity is high at emergence under long-day conditions and remains high in order to initiate reproduction (Khan *et al.*, 1982a). Under short-day conditions, the corpora allata are moderately active at emergence and gradually become more inhibited if the beetles remain in short-days. As a result, the JH titer decreases after emergence, which leads to initiation of the short-day program with an increase of JHE-mRNA on day 2 (Fig. 3b), followed by induction of mRNA encoding Dp-1 on day 3. (de Kort *et al.*, 1997). The increase in JHE-mRNA precedes the elevation in JHE activity in the hemolymph of beetles reared under short-day conditions (Fig. 3). This apparently leads to a further decrease in JH titer and to further implementation of the diapause program. If the JHE activity in the hemolymph reaches its maximum on day 9, the beetles become committed to enter diapause by digging into the soil between days 11-12.

Treatment with JH analog in the early phase of the diapause program can easily reverse it into the reproductive program. Thus, application of pyriproxyfen on day 1 prevents not only the increase in JHE-mRNA and JHE activity of the hemolymph, but also the appearance of mRNA for Dp-1 (de Kort *et al.*, 1997). This reversal can also be concluded from the appearance of the Vg subunits in the hemolymph two days after JH analog application (de Kort *et al.*, 1997) and the females showing mating behavior and oviposition. The reversal is temporary, probably because the JH analog will be endogenously degraded, absorbed or excreted. It takes at least 8 days to decrease the hemolymph JH analog titer below a certain threshold after which the diapause program is resumed (Fig. 5, SD1). The first sign for resumption is the increase in JHE-mRNA on day 9, followed by elevation of JHE activity in the hemolymph thereafter. The first indication that the JH titer has dropped low enough to activate the gene for Dp-1 occurs on day 16 (de Kort *et al.*, 1997). Thus, it seems that for the full expression of the Dp-1 gene a lower JH titer is required than for activation of the JHE gene. Perhaps elevated JHE activity is a prerequisite for such low JH titers.

Application on day 4 induces less dramatic effects than treatment on day 1. The diapause program has already started, but the JH analog still had a reversal effect. The level of JHE-mRNA decreased rapidly (Fig 5b), but the effect on the gene for Dp-1 is less pronounced. Dp-1 disappears from the hemolymph and the Dp-mRNA level decreases gradually (de Kort *et al.*, 1997). Vg appears in the hemolymph, but completion of oocyte development and oviposition was not

observed. From the results illustrated in Figure 5, it can be concluded that it takes another 8 days before the diapause program is resumed. Increase in JHE-mRNA and JHE activity levels are the first signs for resumption of the diapause program.

Once short-day females have entered diapause, the response to JH analog is completely different. Application of JH or JH analog at this phase results in enhancement of JHE activity in the hemolymph. The beetles have been programmed to maintain low JH titers and any elevation of the titer will enhance JHE activity, probably as an attempt to maintain the state of diapause (Kramer, 1978b). Thus, the response of the fat body is complicated and depends on the physiological state of the tissue. If the fat body functions as a storage tissue (during diapause), JH analog induces the synthesis of JHE, but if the fat body still synthesizes hemolymph proteins (during pre-diapause) it suppresses the JHE gene.

In this connection it is important to point out that pyriproxyfen cannot be degraded by JHE, because it does not contain an ester group which can be hydrolyzed by this enzyme. Pyriproxyfen acts as a JH mimic showing all the known effects of JH in larvae and adults. It also induces JHE in the hemolymph, but it will be degraded or absorbed by a, yet unknown, mechanism. The fact that removal of pyriproxyfen from hemolymph and target tissue takes place can be inferred from the observation that after some time (8-15 days) the diapause program is resumed.

In conclusion, JHE activity in the hemolymph of the Colorado potato beetle is necessary for the onset of pupation and diapause. Regulation of JHE gene expression is dependent on the developmental status of the beetle, the external factor photoperiod and the titer of the endogenous substrate JH. Regulation of the JHE gene most likely acts at the transcriptional level. Further studies using cell culture systems with JHE gene promoter constructs will shed light on the regulation of JHE transcription.

CHAPTER 6

Baculovirus expression of two putative juvenile hormone esterase subunits of the Colorado potato beetle

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C.A.D de Kort and J.M. Vlak

ABSTRACT

Two putative juvenile hormone esterase (JHE) genes from the Colorado potato beetle, *Leptinotarsa decemlineata,* have been amplified by reverse transcription PCR and cloned into a baculovirus expression vector, *Autographica californica* multiple-nucleocapsid nucleopolyhedrovirus (AcMNPV). Expression of two putative JHE genes was performed in *Spodoptera frugiperda* cells, either separately or by co-infection. JHE transcripts were detected by Northern analysis with a JHE-cDNA fragment as probe. SDS-PAGE confirmed the presence of a 57 kDa protein, most likely representing the JHE.A and JHE.B forms of a putative JHE subunit. However, juvenile hormone esterase activity was not detected in the media or in extracts of cells. The lack of enzyme activity can be due to multiple factors including incorrect processing of an active beetle JHE dimer by the lepidopteran cells

INTRODUCTION

Juvenile hormone esterase (JHE) of the Colorado potato beetle, *Leptinotarsa decemlineata*, is the main enzyme degrading juvenile hormone (JH) (Kramer *et al.*, 1977). JH regulates metamorphosis and reproduction in insects (Riddiford, 1994; Wyatt & Davy, 1996; de Kort & Granger, 1996). In the Colorado potato beetle, high activities of JHE coincide with a decline of the JH titer. Peaks of JHE in the hemolymph precede pupation of larvae and the onset of diapause in adults (Kramer & de Kort, 1976a; Kramer *et al*, 1977; Vermunt *et al.*, 1997a). The JHE enzyme of the Colorado potato beetle is a dimer consisting of two subunits of 57 kDa, which are linked other than by disulphide bridges (Vermunt *et al.*, 1997a). A cDNA encoding a 57 kDa subunit was cloned and sequenced (Vermunt *et al.*, 1997b). The deduced amino acid sequence was distinct from lepidopteran JHEs such as *Heliothis virescens* (Hanzlik *et al.*, 1989; Venkataraman *et al.*, 1994). RT-PCR and analysis of genomic DNA indicated the existence of two JHE–related genes, JHE.A and JHE.B, which were 77% identical on the basis of amino acid sequence (Vermunt *et al.*, 1998). JHE-mRNA levels in the fat body correlated positively with levels of JHE activity in the hemolymph (Vermunt *et al.*, 1999).

To obtain definitive evidence that the two cloned genes, JHE.A and JHE. B, are indeed coding for JHE, the open reading frames of both genes were amplified with RT-PCR, inserted into a baculovirus vector and expressed in *Spodoptera frugiperda* (Sf21) cells (King & Possee, 1992). The transcription of the two JHE-related genes was analyzed by Northern hybridization, the protein composition by SDS-PAGE and the presence of JHE-activity by an enzyme assay. For comparison Sf21 cells were also infected with a recombinant baculovirus containing JHE from *H. virescens* (Hammock *et al.*, 1990; Roelvink *et al.*, 1992).

MATERIALS AND METHODS

Insects

Larvae and adults of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), were reared on fresh potato foliage as described previously (de Kort *et al.*, 1997).

RT-PCR

Reverse Transcription PCR (RT-PCR) was used to amplify the complete (1.6 kb long) open reading frames of two JHE-related genes, JHE.A and JHE.B (Vermunt *et al.*, 1997b, 1998). Messenger RNA was isolated from fat bodies of 2-day-old 4th-instar Colorado potato beetle larvae with a mRNA isolation kit (Pharmacia). First strand cDNA was synthesized with the First-Strand cDNA Synthesis kit (Pharmacia) and using oligo(dT)$_{18}$ to prime the first strand. Two µl of an 8-times diluted first strand cDNA reaction mixture was used as template for PCR.

PCR was performed in a volume of 50 µl containing *Taq* buffer (Gibco BRL), 2.5 mM MgCl$_2$, 0.2 mM dNTPs, 25 pmol of each primer and 1.25 U *Taq* DNA polymerase (Gibco BRL). Primers (Pharmacia) were derived from the cDNA sequence of JHE

74

from the Colorado potato beetle (Vermunt et al., 1997b; 1998). The sequences of the primers to amplify the JHE coding sequences of 1.6 kb were: est-12, 5' GATCATTTTTCAGGTGTCAATTG 3' (reverse) and
est-13, 5' ATGGCATCCAATCAAAGATAC 3' (forward).

The thermal cycle profile for PCR amplification was as follows: 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 53°C, 1 min at 72°C; 5 min at 72°C using a Thermal cycler (Hybaid). The resulting PCR products of 1.6 kb were analyzed by 1% agarose gel electrophoresis, purified by electro-elution and cloned into pT7Blue (Novagen) for further analysis.

Construction of baculovirus transfer vector and generation of recombinant baculovirus.

The XbaI site of the cloning vector pT7Blue containing the JHE-related cDNAs was changed into a BamHI site (Sambrook et al., 1989). The 1.6 kb JHE fragments (JHE.A and JHE.B) were cloned into the BamHI site of the transfer vector pAcJR1 (Zuidema et al. 1990), which contains pUC8 sequence and polyhedrin flanking sequences of AcMNPV. The constructs were analyzed for correct orientation of the inserts by restriction enzyme analysis by using the asymmetric EcoRI and SalI sites in the JHE coding sequences (Vermunt et al., 1998). The transfer vectors containing the JHE-related cDNAs were co-transfected with linearized AcMNPV DNA (BaculoGold, PharMingen) into Sf21 cells using lipofectin (Gibco BRL; Groebe et al., 1990). The cells were cultured in Hink's medium supplemented with 10% fetal bovine serum (Hink, 1970). The transfection supernatant was subjected to a plaque assay. Viral plaques were screened for the absence of polyhedrin by using an inverted microscope (Leitz Labovert). Recombinant polyhedron-negative viruses were plaque purified and the concentration of recombinant virus was estimated by an end point dilution assay (King & Possee, 1992).

Expression of recombinant baculovirus DNA

To synthesize recombinant JHE, Sf21 cells were infected with plaque-purified recombinant virus at a multiplicity of infection (MOI) of 5 $TCID_{50}$ units/cell for each virus. The cells were cultured in serum-free IPL41 medium when recombinant JHE was needed to be analyzed for protein analysis. As a positive control, infection was also performed with AcPR1, a recombinant baculovirus containing JHE of *H. virescens* which expresses JHE activity in Sf21 cells at high levels (Hammock et al., 1990; Roelvink et al., 1992). As a negative control, cells were infected with wild type AcMNPV DNA (BacPAK5, Clontech).

RNA and protein analysis

Cells and cell media were separated 48 h post-infection by centrifugation for 5 min at 1000 rpm. The cell pellet was washed with and resuspended in phosphate buffered saline (PBS). To analyze JHE mRNA levels in the cells, Northern hybridization was carried out (Sambrook et al., 1989). Resuspended cells were added to TRIzol reagent (Gibco BRL) for total RNA extraction according to the instructions of the manufacturer. RNA was precipitated from the aqueous phase with isopropanol,

rinsed with 75% ethanol and dissolved in water. The RNA concentration was determined with a spectrophotometer (Gene-Quant, Pharmacia) at 260 nm. Five µg samples of total RNA were transferred to Hybond N+ membranes (Amersham) using a Bio-dotSF microfiltration apparatus (BioRad). Hybridization was carried out at 65°C with $[\alpha-^{32}P]$dATP labeled (Gibco BRL) 1.3 kb JHE cDNA fragment (Vermunt et al., 1997b) under stringent conditions (last wash in 0.1 SSC, 0.1% SDS at 65°C) according to standard procedures (Sambrook et al., 1989). After hybridization with the JHE cDNA, the membrane was deprobed and hybridized again with and actin probe (gift of Dr. M.H. Roos, ID-DLO, Lelystad, The Netherlands; Files et al., 1983) for the presence of equal amounts of total RNA on the blot.

Proteins in the medium and in the cells were analyzed by 8.5% SDS-PAGE (Laemmli, 1970) using a Bio-Rad Protean II slab cell. Staining was done with 0.1% Coomassie Brilliant Blue R-250 in 40% methanol, 10% acetic acid, and destaining in 25% ethanol, 9% acetic acid.

Enzyme and protein assays

Enzyme activities were determined from cell media and ultrasonically homogenized cells. After centrifugation of the homogenate, the soluble fraction was used for the assays. JHE activity was measured according to a partition assay (Hammock & Sparks, 1977), as modified by Vermunt et al. (1997a). Racemic $[^{3}H]$-JH III (Amersham) mixed with unlabeled JH III (Calbiochem) at a final concentration of 5 x 10^{-6} M was used as substrate. Undegraded JH was extracted with iso-octane. The aqueous phase containing JH-acid was counted for radioactivity in a liquid scintillation counter (Beckman LS 6000TA) and the amount of hydrolysed JH III was calculated.

A general carboxyl esterase assay was performed according to van Asperen (1962) with α-naphthyl acetate as substrate. The activity was measured spectrophotometrically at 600 nm after staining the hydrolysis product α-naphthol with diazoblue-sodium lauryl sulphate. Protein concentrations were determined according to Bradford (1976) with the Bio-Rad protein assay using bovine serum albumin as a standard. Enzyme activities and protein concentrations were determined in duplicate.

RESULTS

Construction recombinant baculovirus

To express the putative JHE from the Colorado potato beetle in a baculovirus-insect cell culture system, the coding regions of two JHE-related cDNAs, JHE.A and JHE.B (Vermunt et al., 1997b; 1998), were amplified with RT-PCR and first ligated into the cloning vector pT7Blue. The sequences were checked by restriction enzyme analysis for an initial screening and by nucleotide sequencing for further conformation. Both open reading frames were recloned into a baculovirus transfer-vector pAcJR1 (Zuidema et al., 1990). The genes were inserted downstream of the strong polyhedrin promoter. The resulting recombinant transfer vectors were screened for the sense orientation of the inserts and for the presence of the two JHE-related genes by restriction enzyme analysis (Fig. 1). Two vectors, each containing one of the two JHE-related genes, were used to obtain recombinant baculoviruses by homologous recombination of the polyhedrin flanking sequences present in both the transfer vector and the linearized baculovirus DNA. In the resulting recombinant viruses, the JHE-related genes replaced the polyhedrin gene, which were under control of the polyhedrin promoter. Viruses were screened for the absence of polyhedra and plaque purified. The recombinant AcMNPV containing JHE.A was designated AcMU38 and with JHE.B, AcAV1.
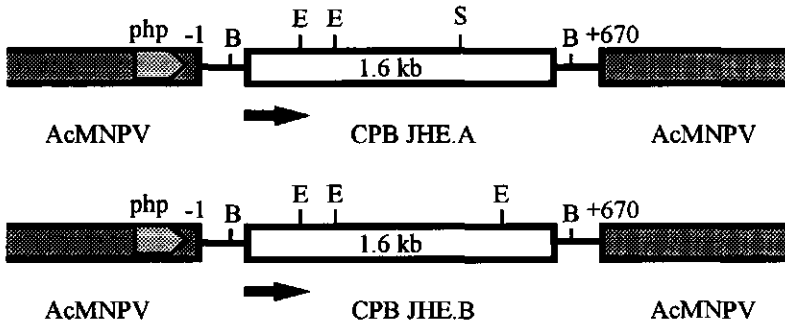
Fig. 1. Schematic representation of recombinant transfer-vectors provided with the coding sequences (1.6 kb) of the JHE.A and JHE.B genes from the Colorado potato beetle (Vermunt et al., 1998) cloned into the BamHI site of pAcJR1 (Zuidema et al., 1990) containing pUC8 plasmid sequence and polyhedrin flanking sequences of AcMNPV. The orientation of the JHE coding sequences were checked by restriction enzyme analysis and indicated by an arrow. The inserted sequences are under control of the AcMNPV polyhedrin promoter (php). CPB JHE.A and JHE.B, putative juvenile hormone esterase genes of the Colorado potato beetle; AcMNPV, Autographica californica multiple-nucleocapsid nucleopolyhedrovirus; -1 and +670 are nucleotide positions relative to the translational start of the polyhedrin gene (the nucleotides in between are replaced by the JHE genes); B, BamHI; E, EcoRI; S, SalI.
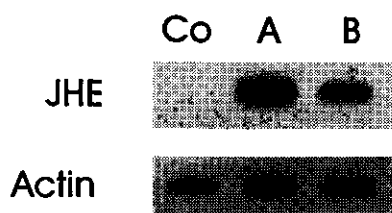
Fig. 2. Analysis of the transcription of JHE-related genes in a baculovirus-insect cell system. Slot blot hybridization of a 1.3 kb ^{32}P-labeled JHE cDNA probe to 5 µg of total RNA extracted from Sf21 cells, uninfected control (Co), infected with AcMU38 (A, recombinant baculovirus containing JHE.A) and infected with AcAV1 (B, recombinant baculovirus containing JHE.B). As internal standard for loading equal amounts of RNA, the same membrane was deprobed and hybridized with an actin probe.
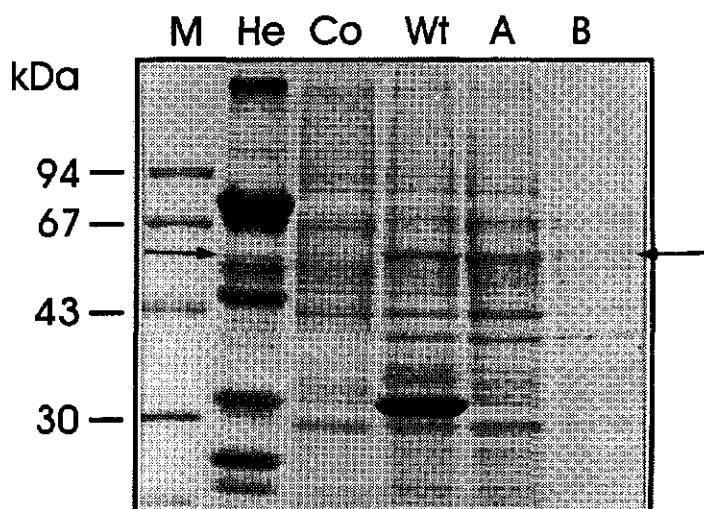
Fig. 3. Analysis of the expression of a 57 kDa protein in the baculovirus-insect cell system. Proteins were separated by SDS-PAGE using 8.5% polyacrylamide gels and stained with Coomassie. Samples: cell lysate of Sf21 cells, uninfected (Co); infected with AcMU38 (A) and infected with AcAV1 (B); infected with wild type (Wt) baculovirus AcMNPV (PAK5); He, hemolymph of 3-day-old 4th-instar larvae with a 57 kDa protein (arrow); M, molecular weight marker.

Expression analysis of JHE in recombinant baculovirus-infected Sf21 cells

Sf21 cells were infected with AcMU38 and AcAV1, encoding the JHE.A and JHE.B, with a MOI of 5 $TCID_{50}$ units/cell. The cells were cultured in serum-free IPL41, harvested 48 h after infection, and resuspended in PBS. Samples of this cell suspension and of the cell culture medium were then analyzed by Northern slot-blot hybridization using a 1.3 kb ^{32}P-labeled JHE cDNA probe (Fig. 2), by SDS-PAGE followed by Coomassie staining (Fig. 3) and by JHE assay (Table 1).

The JHE-specific transcripts were clearly detected in AcMU38 (JHE.A)- and in AcAV1 (JHE.B)-infected cells (Fig. 2). No signal was found in uninfected cells. This result indicated that the correct recombinants were generated and selected. The 57 kDa JHE subunit from the hemolymph of the Colorado potato beetle (arrow, lane He, Fig. 3) was also detected in AcMU38- and AcAV1-infected Sf21 cells, but at a relatively low level (Fig. 3, lanes A and B, respectively). The genes were under control of the polyhedrin promoter and a stronger expression could be expected. The strong 30 kDa polyhedrin band in wild type-infected cells (lane Wt, Fig. 3) is an indication of the strength of the promoter. A protein band just above the putative 57 kDa JHE-subunit was also visible in negative controls (uninfected and wild type AcMNPV-infected cells). This larger protein is obviously distinct from the putative JHE subunit and is a protein of Sf21 cells. The absence of the 30 kDa polyhedrin band in the samples infected with both recombinants confirmed that these viruses were indeed recombinants. When the media from cells infected with the recombinants were analyzed on protein composition, no 57 kDa protein could be detected with Coomassie staining (data not shown).

The media and cells were assayed for JHE activity. In both, cell homogenates and cell media, JHE activity was absent (Table 1). Neither by infection with single recombinants nor by co-infection with both recombinants simultaneously, JHE activity could be detected (Table 1). The latter experiment derived from the hypothesis that only the dimer, maybe a hetero-dimer of JHE.A and JHE.B, has JHE activity (Vermunt et al., 1997a, 1998). The positive control, cells infected with AcPR1 containing JHE of *H. virescens* (Roelvink et al., 1992), showed typical JHE activity. General carboxyl esterase activity was present in AcMU38- and AcAV1-infected cells at the same background level as uninfected cells (data not shown), indicating that the infected cells did not generate additional general esterase activity above the activity which was already present in uninfected cells (data not shown).

Table 1. JHE activity in Sf21 cells, 48 h after infection with recombinant baculoviruses, AcMU38 and AcAV1, containing two JHE-related genes of the Colorado potato beetle, CPB JHE.A and CPB JHE.B respectively. The cells were also co-infected by both recombinants simultaneously. Negative control, Sf21 cells non-infected; positive control, cell media after infection with AcPR1 containing JHE of *H. virescens* (*H. vir.* JHE) (Roelvink *et al.*, 1992); n.d., not determined.

Virus	JHE activity(nmol/min/ml)	
	media	cells
no virus	0	0
AcPR1 (*H. vir.* JHE)	17.6	n.d.
AcMU38 (CPB JHE.A)	0	0
AcAV1 (CPB JHE.B)	0	0
AcMU38 + AcAV1 (CPB JHE.A+JHE.B)	0	0

DISCUSSION

Two recombinant baculoviruses were constructed, containing JHE.A and JHE.B cDNA from the Colorado potato beetle respectively. The coding regions of these two genes replaced the polyhedrin gene of the wild type baculovirus AcMNPV. After infection of Sf21 cells with these viruses, Colorado potato beetle-specific JHE transcripts were clearly detected in the cells (Fig. 2). To exclude the possibility that the signal is due to residual viral DNA a Northern analysis after separation of RNA in agarose gels should be performed. A 57 kDa protein, a size similar to native JHE subunit, was produced in the cells, but at low levels (Fig. 2). JHE activity of the Colorado potato beetle-specific JHE activity could apparently not be generated in this expression system (Table 1).

Compared to the strong expression of polyhedrin in the Sf21 cells, the expression of the two JHE-related genes was weak (Fig. 2). A reason for this may be an inhibition of JHE protein synthesis. Translational control is an important regulator of JHE synthesis, for example in *Heliothis virescens* after polydnavirus infection (Shelby & Webb, 1997) and this may be also the case in Sf21 cells. Alternatively, additional factors, which are unknown at this point, are needed to obtain a full translation of the beetle JHE in lepidopteran cells. On the other hand, high level of recombinant JHE from *H. virescens* could be obtained using the baculovirus-insect cell expression system.

An explanation for the lack of beetle JHE activity is that the inserted JHE genes might contain mutations in the sequence. The recombinant transfer vector was only checked by restriction enzyme analysis and not by sequencing. A further possibility is that the 57 kDa proteins produced were incorrectly processed post-translationally. For example, the biosynthesis of collagens by the baculovirus-insect cell system required processing by up to eight specific post-translational enzymes (Lamberg *et al.*, 1996). Correct glycosylation and acetylation are important factors, which are necessary for the generation of an active enzyme. The lepidopteran Sf21 cells may not possess factors needed for the formation of an active beetle JHE. The esterase is only active as a dimer (Vermunt *et al.*, 1997a). Whether the dimerization of the subunits needs a third factor remains unknown. Finally, it is also possible that a conformational change of a self-associated dimer is needed for the activation of the enzyme.

The JHE transcript and protein were detected in the cells, but the expressed protein was not found in the media by Coomassie staining, indicating that it was not secreted. In contrast, baculovirus expressed *H. virescens* JHE protein was detected in the media by Western blot analysis and 95% of the JHE activity was found in the media (Roelvink *et al.*, 1992). Because the complete open reading frame of the beetle JHE was inserted in the baculovirus, it should contain a signal peptide, which is necessary for secretion. N-terminal analysis of the recombinant, intracellular beetle JHE should clarify this point. The fact that the protein was not found in the medium may be an indication that it was not processed correctly to allow secretion and activation. Maybe, the cleavage of the signal peptide was not performed well.

The possibility that the transcribed genes, JHE.A and JHE.B, are coding for general carboxyl esterases instead of a JH-specific esterase can be ruled out, because during purification of JHE (the preceding step of cloning JHE), the JHE activity was clearly separated from the α-naphthyl acetate esterase activity (Vermunt *et al.*, 1997a). Consequently, the amino acid sequences of the endoproteolytic peptides of the purified enzyme, which were used for the construction of degenerate primers to clone JHE, cannot originate from general carboxyl esterases. Besides, enhanced α-naphthyl acetate esterase activity was not detected after expression of the recombinant baculoviruses in the Sf21 cells.

Ward *et al.* (1992) obtained expression of enzymatically active *H. virescens* JHE in an *E.coli* expression system, using a modified pGEM5Zf(-) plasmid. The expression, however, was poor. Likewise, we inserted the open reading frames of the beetle JHE.A and JHE.B genes downstream of the lacZ promoter in pT7Blue and induced transcription by adding IPTG. However, JHE activity was not detected in the cells or in the media by using this *E. coli* expression system (data not shown).

Sf21 cells infected with AcPR1 containing JHE of *H. virescens* (Roelvink *et al.*, 1992) did express JHE activity, while the JHE-related genes of the Colorado potato beetle did not. The failure to express the latter might be due to several distinct features of JHE from the Colorado potato beetle as compared to JHEs from Lepidoptera. One of these is that JHE activity of the beetle is sensitive to Triton X-100 inhibition, in contrast to lepidopteran JHEs (Kramer & de Kort, 1976b; Kramer *et al.*, 1977, Hammock, 1985). Additionally, the enzyme is a dimer instead of a

monomer (Vermunt *et al.*, 1997a) and its amino acid sequence showed only a limited homology to some functional motifs in JHEs in other species (Ward *et al.*, 1992; Cygler *et al.* 1993; Vermunt *et al.*, 1997b). These different characteristics may be responsible for the failure of synthesis of active beetle JHE in lepidopteran cells. A consideration for further experiments might be the use of a beetle cell line. The chance for correct translation and post-translational modifications such as glycosylation, acetylation, cleavage of signal peptide, folding and assembly of an active dimer might be higher with an expression system that is closer to the organism from which the expressed genes were originally derived.

CHAPTER 7

General discussion

Daylength-dependent expression of juvenile hormone esterase gene in the Colorado potato beetle

The Colorado potato beetle reproduces during the summer. To survive the unfavorable winter, the beetle has developed an adaptation mechanism, called diapause. This physiological state is characterized by lack of development or reproduction and by a low rate of metabolism (de Kort, 1990). It enables insects to survive long periods of lack of food and low temperature during winter. Diapause in the Colorado potato beetle occurs in the adult stage, when the beetle digs into the soil after a period of active feeding (pre-diapause), during which it prepares itself for entering diapause.

The environmental stimulus daylength signals neurosecretory cells in the brain to release peptides. These peptides stimulate or inhibit the corpora allata to synthesize and release juvenile hormone (JH). A low titer of this hormone mediates the daylength-dependent process diapause. Under short-day conditions, JH synthesis is inhibited and consequently the JH titer in the hemolymph drops. During the decrease, a developmental program is implemented, ultimately leading to diapause. First, the JH titer decreases, which already occurs before adult emergence (de Kort, 1990). On day 2 after emergence, the juvenile hormone esterase (JHE) gene is expressed (Chapter 5; Fig. 1). The JHE enzyme degrades JH and decreases the JH titer further. When the JH titer is at a low level, on day 3, the Diapause protein-1 (Dp-1) is expressed (de Kort et al., 1997; Fig. 1). Dp-1 is an arylphorin-type storage hexamer of the beetle. It accumulates in the hemolymph of the last larval instar and (pre-)diapausing adults (Koopmanschap et al., 1992). The sensitivity of Dp-1 gene expression to JH was demonstrated by topical application of 1 µg of the JH analog pyriproxyfen, which suppressed the Dp-1 mRNA levels completely (de Kort et al., 1997). When applied at an early stage, also the JHE gene will be suppressed by this treatment (Chapter 5). At that moment, the beetle is not yet committed for diapause and by JH analog application it can be reprogrammed to follow the 'long-day' program of reproduction. Under long-day conditions, female beetles start to express the vitellogenin (Vg) gene on day 2, which provides the protein reserves for oocyte development (de Kort et al., 1997).
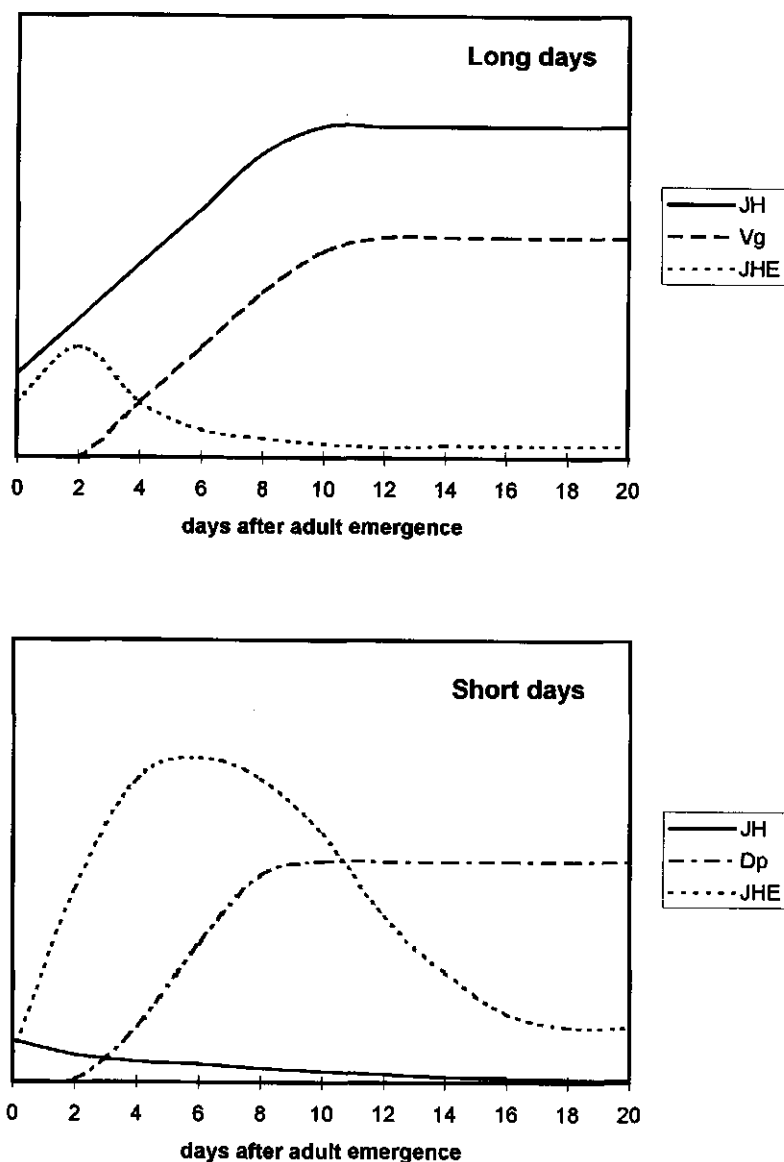
Fig. 1. Schematic representation of several physiological changes in the hemolymph of the adult Colorado potato beetle during photoperiod-dependent development. Relative gene expression of JHE, vitellogenin (Vg) and diapause protein-1 (Dp-1) and JH titer in relation to age, under long-day (top) and short-day conditions (bottom) (data from de Kort (1990), de Kort *et al.* (1997) and Vermunt *et al.* (1999)). Long-day females show, 5-6 days after adult emergence, oviposition. Short-day beetles enter diapause after 11-12 days by digging into the soil.

As compared to other physiological parameters, the advantage of following gene expression by measuring specific mRNA levels, is the accurate determination of the start of a developmental program. For instance, Dp-1 and Vg gene expression can be considered as markers for diapause and vitellogenesis, respectively (de Kort et al., 1997). JHE-mRNA levels are interesting to follow because the JHE enzyme influences the JH titer, which is a determining factor for a number of physiological processes. In addition, JH itself also affects the JHE expression. Feedback regulation was observed in larvae and adults. In the first few days of the last larval instar, JH enhances JHE followed by a decrease in JH titer to allow induction of pupation by ecdysteroids (Hammock, 1985; Chapter 1). Enhancement of JHE by JH has also been observed in diapausing adults (Kramer, 1978b). When treated with JH analog, diapausing beetles will induce JHE. JH application will lead to enhanced JHE to keep the JH titer low, necessary for maintaining diapause. In contrast, if short-day beetles are not yet committed for diapause, JH analog treatment can reprogram the beetles in 'long-day' reproductive animals with low JHE levels.

Because JH application in vivo gives different effects depending on the state of the animal, these effects on the JHE expression cannot be directed by JH alone. Other factors are also involved. Between JH application on the surface of the beetle and the effect on gene expression, many side effects can be induced which can influence the gene of interest. JH can affect factors in various tissues, which in turn can suppress or enhance the JHE gene expression in the fat body. In this connection it is interesting to study the JH signal transduction (Riddiford, 1994; Jones, 1995; Jones & Sharp, 1997), to identify the JH receptors, the second messengers if present, the transcription factors involved and the responsive elements in promoters of affected genes.

We used a fragment of the putative JHE cDNA (Chapter 3) as probe to follow JHE-mRNA levels in the fat body during development of the beetle, under long-day and short-day conditions as well as after treatment with the JH analog pyriproxyfen (Chapter 5). The JHE-mRNA levels in the fat body were positively correlated with JHE activities in the hemolymph. This is a strong indication that the cloned cDNA indeed codes for hemolymph JHE. JHE-mRNA and JHE activity followed a very specific pattern, which is clearly different from for example other JH-dependent genes, such as Dp-1. We have evidence that two JHE-related genes, JHE.A and JHE.B, exist in the Colorado potato beetle and that both are transcribed (Chapter 4). The probe used to follow JHE-mRNA levels was a fragment of JHE.A, 1.3 kb long, and hybridized with JHE.A as well as JHE.B (Chapter 6). Whether JHE.A and JHE.B have different gene expression patterns, we do not yet know. Shorter fragments, which map in an area with high divergence, may be good candidates for use as probes to distinguish gene expression of JHE.A and JHE.B in future experiments.

Structure of JHE and its gene

To follow JHE gene expression in the Colorado potato beetle, the JHE gene had to be cloned. The strategy followed was to successively purify the enzyme, sequence the peptide fragments, construct degenerate primers, perform RT-PCR on mRNA and screen a cDNA library (Chapter 3). For enzyme purification a stage was selected with the highest JHE specific activity (nmol/min/mg protein). This was day 3 of the last larval instar. Hemolymph of these animals was used to purify JHE (Chapter 2). The approach using affinity column chromatography and transition state inhibitors (Abdel-Aal & Hammock, 1986) proved to be inadequate for purification of JHE from the Colorado potato beetle. During pilot experiments we found that JH-specific esterase could be separated from general carboxyl esterases and other major hemolymph proteins by nondenaturing PAGE. JHEs from other insects eluted together with general carboxyl esterases by using this technique (Hammock, 1985). JHE from the beetle separates from general carboxyl esterases during native PAGE because its molecular mass is about twice as high as JHE from other insect species. JHE from the beetle is composed of two subunits of 57 kDa whereas the JHEs of Lepidoptera are monomers of about 65 kDa (Chapter 2). After preparative nondenaturing PAGE with the PrepCell, the JHE-containing fractions were subsequently subjected to isoelectric focusing (pH gradient of 4 to 6.5) (Chapter 2). The JHE activity was focused at two pI's (5.5 and 5.6). In other animals more than one isoform were observed as well (Wing *et al.*, 1984; Venkatesh *et al.*, 1990; Zera *et al.*, 1992).

The finding that enzymatically active JHE is a dimer of two similar size subunits is new. Previously described JHEs were characterized as monomers, although preliminary results now indicate the existence of dimeric JHEs in other insects as well (Dr. B.D. Hammock, personal communication). In contrast to the acetylcholine esterase from the Colorado potato beetle, which is also a dimer, the two subunits of JHE are not linked by disulphide bridges (Zhu & Clark, 1994; Chapter 2). It is not yet known how the JHE subunits are held together, possibly by salt links, hydrogen bonds, van der Waals bonds or hydrophobic interactions. The fact that JHE activity can be inhibited by low concentrations of the nonionic detergent Triton X-100 (Kramer *et al.*, 1977) may indicate that hydrophobic interactions are involved in dimerization. Inhibition of JHE activity by Triton X-100 may be due to monomerization of the JHE dimer. The dimeric structure of the beetle JHE gives at least two possibilities for the location of the catalytic and the JH binding site. We have no indications that the 57 kDa monomers show JHE activity. In case of a dimeric enzyme, the catalytic site can be formed at the interface of both subunits. It is also possible that when two inactive monomers aggregate to form a dimer, each subunit will contain an active catalytic site. Considering the hydrophobicity of JH and the expected hydrophobicity on the interface of both subunits, it may be possible that the binding site is positioned on the interface of both subunits and the cleavage of the ester occurs near this site.

The homology among JHE genes of Lepidoptera (*Heliothis virescens* and *Trichoplusia ni*) and other esterases is higher than the homology between *H. virescens* JHE and the putative JHE from the Colorado potato beetle (Chapter 3). This is probably also the explanation why the initial screening of a Colorado potato beetle cDNA library with JHE cDNA from *H. virescens* did not result in isolation of any

positive clones (de Kort & Koopmanschap, personal communication). Purification and internal amino acid sequencing of the enzyme subunit proved to be the ultimate key to clone the encoding cDNA (Chapter 3). Some functional esterase motifs (Ward et al., 1992) were present in the sequence. However, the GXSXG motif, which is present in most carboxyl esterases was absent as such in the putative JHE cDNA sequence from the Colorado potato beetle. This finding suggests a possible distinct catalytic mechanism compared to other carboxyl esterases.

Different inhibition characteristics of the beetle JHE, when compared to other JHEs by its sensitivity to Triton X-100 (Kramer et al., 1976b, 1977) support this hypothesis. If the catalytic site of the dimeric JHE is formed at the interface of both subunits, then it is possible that amino acids from each subunit form one catalytic site, like in some aspartyl proteases (HIV protease and pepsin; Stryer, 1995). In contrast, the dimeric acetylcholine esterase contains a catalytic site on each subunit (Sussman et al., 1991).

The fact that only limited homology was found between lepidopteran JHEs and the putative beetle JHE (Chapter 3) is not surprising if we look to another protein in the JH field: the JH binding protein (JHBP). This protein binds specifically JH with high affinity (dissociation constant in nM range). This protein has been characterized for many insect species and from some the gene has been cloned. Three classes of these proteins are known now, reflected by different subunit architecture and distinct encoding genes. Lepidopteran JHBPs are monomers of around 30 kDa. Orthepteran JHBPs are classified as hexamerins (77 kDa subunits). In other orders, lipophorins (250 kDa and 80 kDa subunits) act as JHBP (Prestwich et al., 1994; de Kort & Granger, 1996). In this connection, it is not surprising that JHEs of different orders are also distinct. Lepidopteran JHEs are similar in amino acid sequence to acetylcholine esterases, while the putative JHE genes of the Colorado potato beetle are not related to any known protein present in databanks (Chapter 3). More JHE genes, especially those from other orders than Lepidoptera, need to be identified, before a classification in JHE genes can be made.

RT-PCR experiments and analysis of genomic DNA showed the existence of two JHE-related genes, JHE.A and JHE.B, which were 77% identical in the amino acid sequence (Chapter 4). Both genes are transcribed in the fat body, as shown by RT-PCR experiments. Both transcripts were present in each individual. The two genes probably arose by duplication of the JHE locus (Chapter 4). Further evidence that the putative JHE genes of the Colorado potato beetle are distinct from lepidopteran JHE genes, is the fact that the genes from the beetle did not contain introns (Chapter 4; Harshman et al., 1994; Venkataraman et al., 1994).

What are the separate functions of the two JHE-related genes in the Colorado potato beetle, JHE.A and JHE.B? JHE.A is probably present in the hemolymph, because three HPLC-separated endoproteinase Lys-C peptides of purified hemolymph JHE were encoded by the cloned cDNA of JHE.A (Chapter 3). In addition, the deduced amino acid sequence of JHE.A cDNA predicted a pI of 5.5, which is very similar to the experimentally determined values of 5.5 and 5.6 for hemolymph JHE (Chapter 2). It is less likely that JHE.B also occurs in the hemolymph. An isoelectric point of 6.9 was predicted for JHE.B. Unless JHE.B is extensively modified after

translation, it probably does not contribute to hemolymph JHE. It is possible however, that JHE.B may act as cytosolic JHE. When necessary, JH should also be purged from tissues in addition to the degradation in the hemolymph, for a quick response. Hemolymph JHE from the Colorado potato beetle is only active as a dimer. If JHE.B is not present in the hemolymph, then consequently hemolymph JHE should be a homodimer consisting of two JHE.A subunits.

To synthesize enough enzymatically active recombinant JHE for structure-function analysis and to obtain definitive evidence that the cloned genes, JHE.A and JHE.B, are indeed coding for a JHE, the open reading frames of both genes were expressed in the baculovirus-insect cell expression system (Chapter 6). The construction of the recombinants and the synthesis of the JHE transcripts were successful, but the amount of translation product was low. However, protein subunits of the correct size, 57 kDa, were produced. This recombinant JHE was not enzymatically active. If this was succeeded, efforts could have been made to improve the activity of baculovirus for insect control by expressing JHE of the Colorado potato beetle via the baculovirus. JHE from *H. virescens* was expressed via a baculovirus vector. This recombinant JHE virus reduced feeding in *Manduca sexta* after infection (Hammock *et al.*, 1990). However, recombinant JHE was rapidly sequestered and degraded. Modifications of the recombinant JHE virus are performed to improve the insecticidal activity (Bonning *et al.*, 1997). One approach is to express a heterologous JHE in a target insect by the baculovirus. This distinct JHE would possibly not be recognized by degradation systems (Hammock *et al.*, 1993). Thus, JHE from the Colorado potato beetle could have been an attractive candidate to control Lepidoptera.

Final evidence for cloning the JHE gene of the Colorado potato beetle was not provided due to failing attempts to synthesize functionally active JHE in a baculovirus-insect cell system. Nevertheless, the tightly positive correlation between mRNA levels of the putative JHE gene(s), followed by using a fragment of the cloned cDNA, and the JHE activity in various conditions strongly indicates that we indeed cloned the JHE gene (Chapter 5). In addition, the sequences of three Lys-C peptides of the purified JHE (Chapter 2) were also encoded in the sequence of the cloned cDNA (Chapter 3) and one Lys-C peptide of 23 amino acids showed 30% identity to some other insect esterases (Chapter 3). It remains possible, however, that a closely related gene is cloned and expressed, which correlates with JHE expression in the beetle. The lack of a typical serine esterase motif (GXSXG) in both JHE genes may suggest that we have cloned a JHE pseudogene, although evidence from genomic and cDNA analysis (Chapter 4) is lacking for this suggestion.

A future challenge in the daylength-dependent gene expression is to shed light on the photoperiodioc clock and how this clock is linked to switching on a certain developmental program by affecting specific genes. The role of JH is evident in this process, in the Colorado potato beetle and many other insects. Detailed study of JHE transcription will provide more insight in the JH titer regulation and can also elucidate the molecular action of JH, either directly on the promoter or indirectly via a signal pathway.

CHAPTER 8

Summary

A number of important physiological processes in insects is controlled by the titer of juvenile hormone (JH). The juvenile (larval) stage is maintained at a high JH titer, whereas the onset of metamorphosis is induced by a low JH titer. Reproduction by adults requires often a high JH titer. Through synchronization of the reproductive phase with favorable environmental conditions, insects are able to adapt to adverse conditions and to enhance the chance for survival. The Colorado potato beetle uses daylength (photoperiod) as key stimulus for seasonal adaptation. In temperate areas the beetle hibernates in the soil and development is arrested. If the adult beetle is exposed to short-day conditions it digs into the soil after 11-12 days, which marks the onset of diapause. This photoperiodic effect is mediated by JH. Long-day conditions result in a high JH titer leading to reproduction. Short-day conditions, however, result in a low JH titer leading to diapause. The JH titer in the hemolymph is the result on the one hand by JH synthesis in the corpora allata and on the other hand by JH degradation in the hemolymph. Hydrolysis of the JH ester by JH esterase (JHE) is the main JH degradation pathway in the beetle. The highest JHE activity occurs in the hemolymph (Chapter 1).

Surprisingly, the JHE from the Colorado potato beetle (Coleoptera) was found to be a dimer, consisting of two subunits of 57 kDa, whereas lepidopteran JHEs were characterized as monomers. The fact that the beetle JHE occurs as a dimer, is also the reason why the enzyme could be separated from monomeric general carboxyl esterases by nondenaturing polyacrylamide gel electrophoresis (PAGE). After PAGE purification, narrow-range isoelectric focusing and SDS-PAGE were employed to purify JHE from hemolymph of the last larval instar of the Colorado potato beetle. Two forms were found with isoelectric points of 5.5 and 5.6, respectively (Chapter 2).

To clone the encoding cDNA, the purified JHE was subjected to endoproteinase Lys-C digestion. Based on the amino acid sequence of the separated peptides, degenerate primers were designed to perform RT-PCR in order to clone an internal fragment of the encoding cDNA. The 3'-end was found by screening a cDNA library and the 5'-end by using the 5'RACE technique. The size of the transcript was 1.7 kilobase. The deduced amino acid sequence (515 residues) of the putative JHE cDNA showed limited homology to some functional peptide motifs of other insect esterases (Chapter 3).

RT-PCR on mRNA and analysis of genomic DNA provided evidence for the existence of two JHE-related genes, JHE.A and JHE.B. Both are intronless in the coding regions. JHE.A with a predicted pI of 5.5 probably codes for hemolymph JHE. The function of JHE.B with a predicted pI of 6.9 is unknown yet, but it may code for cytosolic JHE in the fat body. Both genes lack a conserved motif with a catalytic

serine, typical for serine esterases. It was hypothesized that dimerization of the 57 kDa subunits can generate a catalytic site for enzyme activity (Chapter 4).

In the beetle, the JHE transcript was mainly synthesized during the feeding stage of the last larval instar as well as in pre-diapausing animals (short-day adults). At these stages the JH titer is low and induces pupation and diapause, respectively. JHE activity in the hemolymph correlated with JHE-mRNA levels in the fat body during the development of the beetle. This observation is a strong indication that the probe used to follow JHE-mRNA levels indeed codes for hemolymph JHE. Also after photoperiodic and JH analog treatment the positive correlation was found between JHE-mRNA levels and JHE activity. JH analog (pyriproxifen) application to early 4th-instar larvae resulted in a negative feedback as JHE synthesis was enhanced to maintain the JH titer low. In contrast, if the JH analog was applied to early short-day adult beetles, JHE gene expression was suppressed to reprogram the beetles. Instead of preparation for diapause with a low JH titer and a high JHE level, the treated beetles showed reproductive development with a low JHE level to maintain a high JH titer (Chapter 5).

The putative JHE cDNAs, JHE.A and JHE.B, were expressed in a baculovirus-insect cell system. The JHE-specific transcripts were clearly present in the *Spodoptera frugiperda* cells, as detected by Northern hybridization. Also a 57 kDa protein was synthesized, as visualized by Coomassie Brilliant Blue staining, although it was at a low level. However, JHE enzyme activity could not be detected. During translation or post-translational modification, other unknown factors may be required for the formation of an enzymatically active dimeric JHE (Chapter 6).

Finally, following expression of specific genes gives us accurate information about the initiation of a developmental program. In long-day beetles, the JHE gene is switched off and the JH titer increases, leading to reproduction with vitellogenin expression. In contrast, short-days result in switching on of the JHE gene and a low JH titer. This induces the alternative developmental program, diapause, with the expression of diapause proteins. How signal transduction occurs from photoperiodic perception to gene activity is unknown yet (Chapter 7).

SAMENVATTING

Een aantal belangrijke fysiologische processen bij insecten wordt gereguleerd door de concentratie van het juveniel hormoon (JH) in de hemolymfe. Het juveniele (larvale) stadium wordt in stand gehouden door een hoge JH titer, terwijl metamorfose een lage JH titer vereist. Reproductie van de adult gaat bij veel insecten gepaard met een hoge JH titer. Door synchronisatie van de reproductieve fase met gunstige omgevingsomstandigheden, kunnen insecten zich aan ongunstige omstandigheden aanpassen en de kans op overleving verhogen. De Coloradokever, *Leptinotarsa decemlineata* (Say), gebruikt daglengte als signaal voor seizoenssynchronisatie. In gematigde gebieden overwintert de kever in de grond en zijn ontwikkeling verloopt dan heel traag (diapauze). Als de adulte kever wordt blootgesteld aan 'korte-dag' omstandigheden, zal deze zich na ongeveer 11 dagen de grond ingraven, hetgeen het begin van diapauze markeert. Dit daglengte-effect wordt gereguleerd door JH. 'Lange-dag' condities resulteren in een hoge JH titer, hetgeen leidt tot reproductie. 'Korte-dag' condities, echter, resulteren in een lage JH titer, hetgeen leidt tot diapauze. De JH titer in de hemolymfe is de resultante van aan de ene kant JH-synthese in de corpora allata van de kever en aan de andere kant afbraak van JH in de hemolymfe. De hydrolyse van de JH-ester door een JH-esterase (JHE) is de voornaamste JH-afbraakroute in de kever (Hoofdstuk 1).

Het was verrassend dat het JHE van de Coloradokever, een keverachtige, aanwezig is als een dimeer, bestaande uit twee subeenheden van elk 57 kDa. JHEs van vlinderachtigen komen voor als monomeren. Het feit dat het JHE opgebouwd is als een dimeer, verklaart waarom het enzym gescheiden kon worden van monomere, algemene carboxylesterases via niet-denaturerende polyacrylamide-gelelectroforese (PAGE). Na deze PAGE stap, werd isoelectrische focussering met een nauwe pH-gradiënt en SDS-PAGE uitgevoerd om JHE uit de hemolymfe van het laatste larvale stadium van de Coloradokever te zuiveren. Er werden twee vormen gevonden met isoelectrische punten van respectievelijk 5.5 en 5.6 (Hoofdstuk 2).

Om de aminozuurvolgorde van het JHE vast te stellen, werd het coderende cDNA opgespoord. Hiervoor werd het gezuiverde JHE behandeld met endoproteinase Lys-C. Op basis van de aminozuurvolgorde van de resulterende peptiden, werden gedegenereerde 'primers' ontworpen om RT-PCR op kever mRNA uit te voeren om een fragment van het coderende cDNA te kloneren. Het 3'-uiteinde werd gevonden door een cDNA bank van de kever te onderzoeken op de aanwezigheid van JHE-sequenties en het 5'-uiteinde door gebruik te maken van de 5'RACE techniek. De grootte van het JHE-transcript was 1.7 kb. De afgeleide aminozuurvolgorde (515 residuen) van het vermeende JHE cDNA vertoonde een beperkte homologie met enkele functionele motieven van andere insectenesterases (Hoofdstuk 3).

RT-PCR op mRNA en analyse van genomisch DNA suggereerden het bestaan van twee JHE-gerelateerde genen, JHE.A en JHE.B. Beide bevatten geen intronen in het coderende gebied. JHE.A met een voorspelde pI van 5.5 codeert waarschijnlijk voor JHE dat in de hemolymfe voorkomt. De functie van JHE.B met een voorspelde pI van 6.9 is nog onbekend, maar het kan coderen voor een vorm van JHE, die in het

cytosol van cellen in het vetlichaam voorkomt. Beide eiwitten missen een geconserveerd motief met een serine als onderdeel van de catalytische plaats, typisch voor serine esterases. Verondersteld werd dat dimerisatie van de 57 kDa subeenheden een catalytische plaats kan genereren (Hoofdstuk 4).

In de kever werden JHE transcripten voornamelijk gesynthetiseerd tijdens het het laatste larvale stadium wanneer de dieren zich sterk voedden, als ook in dieren die in pre-diapauze verkeerden ('korte-dag' adulten). De JH titer daalt tijdens deze stadia en induceert respectievelijk verpopping en diapauze. JHE-activiteit in de hemolymfe vertoonde een positieve correlatie met JHE-mRNA niveaus in het vetlichaam tijdens de ontwikkeling van de kever. Deze waarneming is een sterke aanwijzing dat de 'probe' die gebruikt werd om de JHE-mRNA niveaus mee te volgen, het gekloneerde cDNA, inderdaad een deel van de code bevat voor hemolymfe JHE. Ook na fotoperiodieke behandeling en een behandeling met een JH-analoog werd een positieve correlatie gevonden tussen JHE-mRNA niveaus en JHE-activiteit. JH-analoog (pyriproxyfen) behandeling van vroege vierde-stadium larven resulteerde in een negatieve terugkoppeling, aangezien JHE-synthese toenam waardoor een lage JH titer gehandhaafd werd. Dit in tegenstelling tot de JH-analoog behandeling van vroege 'korte-dag' kevers, waar JHE genexpressie onderdrukt werd om de kevers te herprogrameren. In plaats van het voorbereiden op diapauze met een lage JH titer en een hoog JHE niveau, lieten deze kevers reproduktieve ontwikkeling zien met een laag JHE niveau waardoor een hoge JH titer werd aangehouden.

De vermeende JHE cDNAs, JHE.A en JHE.B, werden tot expressie gebracht in een baculovirus-insectencelsysteem. De JHE-specifieke transcripten waren aanwezig in de *Spodoptera frugiperda* cellen, aangetoond met Northern hybridisatie. Ook werd er een 57 kDa eiwit gesynthetiseerd, hoewel niet in grote hoeveelheden. Er kon echter geen JHE-enzymactiviteit worden aangetoond. Tijdens translatie en post-translationele modificatie zijn er mogelijk andere, nu nog onbekende factoren noodzakelijk om een enzymatisch actief dimeer JHE te vormen (Hoofdstuk 6).

Het volgen van de expressie van specifieke genen geeft ons nauwkeurige informatie over de aanvang van een ontwikkelingsprogramma. In 'lange-dag' kevers wordt het JHE gen uitgeschakeld en de JH titer stijgt, hetgeen leidt tot reproductie met expressie van dooiereiwitten. Korte dagen, echter, resulteren in het aanschakelen van het JHE gen en een lage JH titer, hetgeen leidt tot het alternatieve ontwikkelingsprogramma, diapauze, waarbij diapauze-eiwitten tot expressie komen. De wijze van signaaloverdracht van fotoperceptie tot genactiviteit is nog onbekend (Hoofdstuk 7).

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98

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Curriculum Vitae

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List of publications

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