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Allelochemical Induction of Hormone-metabolizing Microsomal Monooxygenases in the Fall Armyworm

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Simon J. Yu (2000) Allelochemical induction of hormone-metabolizing microsomal monooxygenases in the fall armyworm. *Zoological Studies* **39**(3): 243-249. Juvenile hormone III was oxidized by midgut microsomes prepared from larvae of the fall armyworm, *Spodoptera frugiperda* (JE Smith). The oxidase is membrane bound, requires NADPH for activity, and is inhibited by carbon monoxide and piperonyl butoxide; it therefore is a typical microsomal monooxygenase, hereafter referred to as JH III oxidase. Feeding the allelochemicals, indole-3-carbinol and β-naphthoflavone, to larvae caused 188% and 105% increases, respectively, in JH III oxidase activity as compared with the controls. Induction of microsomal ecdysone 20-monooxygenase activity by allelochemicals was further studied in this insect. Dietary monoterpenes [(+)-pulegone, (+)-limonene, β-myrcene, (+)-camphor, (+)-camphene, α-terpinene, Γ-terpinene], sesquiterpenes (farnesol, nerolidol), indoles (harmine), coumarins (coumarin), methylenedioxphenyl compounds (safrole, isosafrole, myristicin, piperonyl butoxide), and ketohydrocarbons (2-tridecanone) all stimulated ecdysone 20-monooxygenase activity in larvae from 53% to 234% as compared with the controls. Harmine was the most potent inducer among those tested. Azadirachtin when administered orally or by injection had no effect on ecdysone 20-monooxygenase activity in fall armyworms.

Key words: Juvenile hormone, Ecdysone, Cytochrome P450, Fall armyworm, Allelochemicals.

The microsomal cytochrome P450 monooxygenase system of insects plays an important role in the metabolism of xenobiotics such as insecticides (Nakatsugawa and Morelli 1976) and endogenous compounds such as insect hormones (Feyereisen and Durst 1978, Yu and Terriere 1978). This system can be induced by a variety of chemicals including insecticides, insect hormones, growth regulators, barbiturates, and allelochemicals (Brattsten et al. 1977, Terriere 1984, Yu 1986 1999).

In a previous report (Yu 1995), it was shown that microsomal ecdysone 20-monooxygenase can be induced by indoles (e.g., indole-3-carbinol), flavonoids (e.g., flavone), monoterpenes (e.g., menthol), and coumarins (e.g., xanthotoxin) in fall armyworm larvae. Although juvenile hormone I has been reported to be metabolized by microsomal monooxygenases in several species of insects (Ajami and Riddiford 1973, Hammock et al. 1977, Yu and Terriere 1978), it is not known whether allelochemicals can stimulate the oxidative metabolism of juvenile hormone in phytophagous insects. If such metabolism occurs, it could explain the detrimental effects of numerous allelochemicals on growth and development of phytophagous insects (Reese and Holyoke 1987, Yu 1987).

In the present study, microsomal oxidation of juvenile hormone (JH) III and its induction by allelochemicals was investigated in the fall armyworm. The effect of various classes of allelochemicals on ecdysone 20-monooxygenase activity was further examined in this insect.

MATERIALS AND METHODS

Insects

Larvae of the fall armyworm, *Spodoptera frugiperda*, were reared on an artifical diet and maintained in environmental chambers at 25 °C with a 16:8 light-dark photoperiod as described previously

(Yu 1982).

Chemicals

The chemicals used in this study and their sources were [23,24-^{3}H] ecdysone (specific activity of 54.0 Ci/mmol) and [10-^{3}H(N)] juvenile hormone III (specific activity of 19.5 Ci/mmol) (New England Nuclear, Boston, MA); ecdysone, 20-hydroxyecdysone, indole-3-carbinol, harmine, β-myrcene, farnesol, (+)-camphor, α-terpinene, Γ-terpinene, β-naphthoflavone, and azadirachtin (Sigma Chemical, St. Louis, MO); (+)-pulegone, (+)-limonene, (+)-camphene, nerolidol, coumarin, and safrole (Aldrich Chemical, Milwaukee, WI); myristicin (Saber Laboratories, Morton Grove, IL); and 2-tridecanone, isosafrole, and piperonyl butoxide (Fluka Chemical, Haupauge, NY). All other chemicals were of analytical quality and were purchased from commercial suppliers. JH III diepoxide (bisepoxide), methyl 6,7; 10,11-bisepoxyfarnesoate, was a gift from Dr. C.-M. Yin of the Univ. of Massachusetts, Amherst, MA.

Synthesis of ^{3}H-JH III metabolites

The standards used in cochromatography with in vitro metabolites were prepared from ^{3}H-JH III diluted with cold JH III. Labelled JH III acid (JH_A) was synthesized from JH III according to Goodman and Goodman (1981). Labelled JH III diol (JH_D) was synthesized by the method of Strambi et al. (1981). Labelled JH III diol acid (JH_{DA}) was synthesized from JH III diol according to Goodman and Goodman (1981). Labelled JH III diepoxide (JH_{DE}) was synthesized from JH III by the method of Yin et al. (1995). All products were purified by thin-layer chromatography using EM silica gel 60 F-254 TLC plates. The chromatoplates were developed with hexane/ethyl acetate (100/15, v/v), followed by benzene/ethyl acetate/acetic acid (100/30/3, v/v). The radioactive products were detected by scanning on a Packard radiochromatogram scanner (Model 7220/21). The zone of radioactivity was scraped from the plates and extracted 3 times with 2.5 ml of ethyl acetate for further use in cochromatography.

Treatment of insects

Groups of newly molted 6th instars were fed artificial diets containing various allelochemicals. Controls were fed the artificial diet only. After feeding for 48 h, the larvae were removed from their respective diets and used for enzyme assays. No mortality was observed due to the treatments.

Enzyme preparation

To prepare microsomes for the present study, midguts were dissected from 2-d-old 6th instars, and their gut contents were removed. They were then washed in 1.15% KCl and homogenized in 25 ml of ice-cold 0.1 M sodium phosphate buffer, pH 7.5, in a motor-driven tissue grinder for 30 s. The crude homogenate was filtered through cheesecloth, and the filtered homogenate was centrifuged at 10 000 g for 15 min in a Beckman L5-50E ultracentrifuge. The pellet (cell debris, nuclei, and mitochondria) was discarded, and the supernatant was recentrifuged at 105 000 g for 1 h. The microsomal pellet was rinsed with ice-cold 0.1 M sodium phosphate buffer, pH 7.5, prior to suspension in the same buffer and was used immediately. The above procedures were conducted at 0 to 4 °C.

Protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Enzyme assays

When microsomal JH III esterase and epoxide hydrolase were assayed, the 5-ml incubation mixture contained 1 mg of microsomal protein, 0.1 M sodium phosphate buffer, pH 7.5, 50 μg of cold JH III together with 6.875 ng of ^{3}H-JH III (1.1×10^6 dpm), and 10 mg of bovine serum albumin. Mixtures were incubated in a water bath with shaking for 30 min. The reaction was stopped by saturating the incubation mixture with ammonium sulfate, after which the mixtures were extracted 3 times with 2.5 ml of ethyl ether/ethanol (2/1, v/v). The extracts were combined and dried over sodium sulfate. Aliquots of the extract were then evaporated to approximately 0.2 ml and analyzed by TLC as described above. The appropriate zones of the metabolites (based on cochromatography with known standards) were scraped from the plates and measured for radioactivity by liquid scintillation counting. The above procedure resulted in the recovery of 70% (on the average) of the added radioactivity.

Microsomal oxidation of JH III was determined using the NADPH-dependent substrate disappearance method (Yu and Terriere 1978) since the oxidase is not active without NADPH. The reaction conditions were identical to those with JH III esterase and epoxide hydrolase with the addition of an NADPH-generating system consisting of 1.8 μmol of NADP, 18 μmol of glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase. Incubation mixtures containing no NADPH-generating system

were used as controls. Incubations were conducted as described above. To quantify the unmetabolized JH III after extraction, aliquots of the extract were streaked onto a TLC plate with unlabelled JH III as a marker. The plates were developed as described earlier (Yu 1995). The area corresponding to JH III, as identified under ultraviolet light, was scraped and measured for radioactivity by liquid scintillation counting.

Microsomal ecdysone 20-monooxygenase activity was measured as described previously (Yu 1995). Briefly, the 100-µl reaction mixture contained 0.25 mg of microsomal protein, 0.1 M sodium phosphate buffer, pH 7.5, 1 mM NADPH, 20 ng of ecdysone, and 0.8 ng of [23,24-^{3}H] ecdysone (2.2 x 10^{5} dpm). Reaction mixtures that contained no NADPH were used as controls. Incubations were conducted in a water bath with shaking at 30 °C in an atmosphere of air for 10 min. The reaction was stopped by the addition of 100 µl of ethanol, and the mixture was centrifuged to precipitate the protein. An aliquot of the supernatant was streaked onto a TLC plate as mentioned above with unlabelled ecdysone and 20-hydroxyecdysone as markers. The plate was developed twice in a solvent of chloroform/ethanol (4/1, v/v). Areas corresponding to ecdysone and 20-hydroxyecdysone, as identified under ultraviolet light, were scraped and measured for radioactivity by liquid scintillation counting.

Statistical analysis

Data were analyzed by Student's *t*-test when appropriate.

RESULTS

Fig. 1 shows a typical chromatographic resolution of metabolites produced with in vitro metabolism of ^{3}H-JH III by fall armyworm microsomes. In this experiment, newly molted 6th instars were routinely fed with a diet containing indole-3-carbinol for 2 d prior to preparation of microsomes in order to increase microsomal oxidase activity. The 5 incubation mixtures represented here contained the same levels of substrate (i.e., 50 µg of JH III and 6.875 ng of ^{3}H-JH III). In the absence of NADPH, 3 radioactive zones were observed, JH III, JH III acid, and JH III diol; JH III acid was a minor metabolite. When NADPH was added to the incubation mixture, the level of JH III decreased by about 60% as compared with the control (i.e., without NADPH). In addition, there was an increase in radioactivity in the regions of JH III diol and diol acid. In all instances, JH III diepoxide (an oxidative product of JH III) was not detected. However, when JH III diepoxide was incubated with fall armyworm microsomes, 2 metabolites were produced, which migrated to the positions occupied by JH III diol and diol acid (results not shown). This metabolism of JH III diepoxide would explain an increase in radioactivity in the regions of JH III diol and JH III diol acid seen in the incubation mixture fortified with NADPH, although attempts were not made to identify these 2 metabolites. Addition of

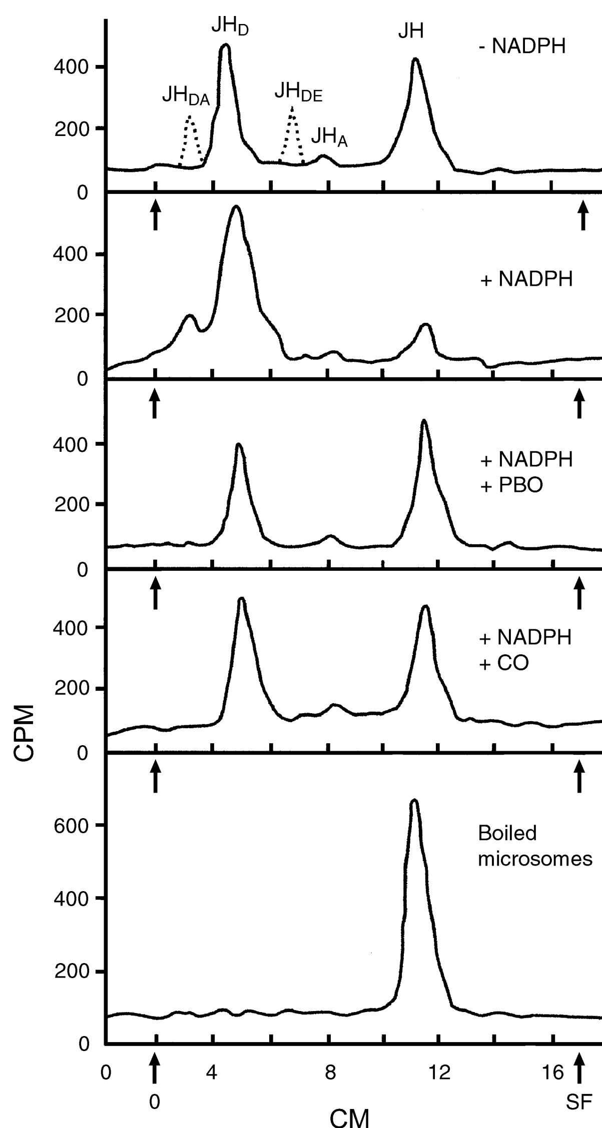

Fig. 1. Typical thin-layer radiochromatogram showing microsomal metabolism of ^{3}H-JH III under different conditions. Subscript letters refer to: A, acid; D, diol; DA, diol acid; DE, diepoxide. The zones occupied by JH$_{DA}$ and JH$_{DE}$ are indicated by the dotted line. 0, origin; SF, solvent front.

piperonyl butoxide (PBO) at 10^{-4} M or CO (bubbled gently for 1 min prior to incubation) resulted in complete inhibition of the oxidative metabolism of JH III, but had no effect on JH III epoxide hydrolase or esterase activity. No metabolism of JH III occurred when boiled microsomes were used as the enzyme source. These results indicate that the NADPH-dependent JH III-metablizing enzyme is a typical microsomal monooxygenase, hereafter referred to as JH III oxidase.

The activities of the 3 JH III-metabolizing enzymes in midgut microsomes prepared from fall armyworm larvae fed on the artificial diet are summarized in Table 1. It can be seen that activity was highest for JH III epoxide hydrolase, followed by JH III oxidase and JH III esterase. There was a 13-fold difference in activity between JH III esterase and JH III epoxide hydrolase.

Data in Table 2 show that JH oxidase can be induced by feeding the larvae with indole-3-carbinol and β-naphthoflavone, with 3- and 2-fold increases, respectively, in activity as compared with the controls. These allelochemicals did not affect the activities of JH III epoxide hydrolase or JH III esterase (data not shown).

Table 3 shows the stimulatory effects of various allelochemicals on microsomal ecdysone 20-monooxygenase in fall armyworm larvae. Monoterpenes

Table 1. JH III-metabolizing enzyme activity in midgut microsomes from fall armyworm larvae

JH III oxidase (nmol/30 min/ mg protein)[a]	JH III esterase (nmol/30 min/ mg protein)[a]	JH III epoxide hydrolase (nmol/30 min/ mg protein)[a]
10.16 ± 1.16	4.22 ± 0.36	57.10 ± 3.73

[a]Microsomes were prepared from midguts of 2-d-old 6th instar larvae. Mean ± S.E. of 3 experiments, each with duplicate determinations.

Table 2. Effect of allelochemicals on midgut microsomal JH III oxidase activity in fall armyworm larvae[a]

Allelochemical	JH III oxidase (nmol/30 min/ mg protein)[b]	Percent of control
Control	9.24 ± 1.22	100
Indole-3-carbinol	26.68 ± 1.74*	288
β-naphthoflavone	18.99 ± 2.16*	205

[a]Newly molted 6th instar larvae were fed artificial diets containing the allelochemicals (0.2%, w/w) for 2 d prior to enzyme assays.
[b]Mean ± S.E. of 3 experiments, each with duplicate determinations.
*Value significantly different from the control ($P < 0.05$).

Table 3. Effect of allelochemicals on midgut microsomal ecdysone 20-monooxygenase activity in fall armyworm larvae[a]

Allelochemical	Ecdysone 20-monooxygenase (pmol/min/mg protein)[b]	Percent of control
Control	0.32 ± 0.04	100
(+)-Pulegone	0.66 ± 0.13*	206
(+)-Limonene	0.49 ± 0.04*	153
β-Myrcene	0.84 ± 0.19*	263
Geraniol	0.39 ± 0.02	122
(+)-Camphor	0.59 ± 0.08*	184
(+)-Camphene	0.43 ± 0.02*	134
α-Terpinene	0.57 ± 0.07*	178
Γ-Terpinene	0.58 ± 0.03*	181
Farnesol	0.77 ± 0.08*	241
Nerolidol	0.69 ± 0.09*	216
Harmine	1.07 ± 0.02*	334
Coumarin	0.60 ± 0.03*	188
Safrole (0.05%)	0.72 ± 0.13*	225
Isosafrole (0.05%)	0.74 ± 0.04*	231
Myristicin (0.05%)	0.63 ± 0.04*	197
Piperonyl butoxide (0.05%)	0.82 ± 0.04*	256
2-Tridecanone	0.54 ± 0.06*	169

[a]Newly molted 6th instar larvae were fed on artificial diets containing the allelochemicals (0.2%, w/w), unless otherwise stated, for 2 d prior to enzyme assays.
[b]Mean ± S.E. of 3 experiments, each with duplicated incubations.
*Values significantly different from the control ($P < 0.05$).

such as (+)-pulegone, (+)-limonene, β-myrcene, (+)-camphor, (+)-camphene, α-terpinene, and Γ-terpinene; sesquiterpenes such as farnesol and nerolidol; indoles such as harmine; coumarins such as coumarin; methylenedioxyphenyl compounds such as safrole, isosafrole, myristicin, and piperonyl butoxide; and ketohydrocarbons such as 2-tridecanone all stimulated ecdysone 20-monooxygenase activity from 34% to 234% as compared with the controls. Harmine was the most potent inducer among those tested. Azadirachtin had no effect on ecdysone 20-monooxygenase activity when larvae were fed with the allelochemical at 2 ppm for 48 h. Similar results were also obtained when 1-d-old 6th instar larvae were injected with this compound at 0.3 μg/larva at 0 h, followed by the same dose at 24 h; enzyme activity was determined at 48 h (data not shown).

DISCUSSION

The results of this study clearly demonstrate that JH III can be metabolized by 3 enzyme systems, namely, an epoxide hydrolase, an oxidase, and an esterase, in midgut microsomes prepared from fall armyworm larvae. Since midgut microsomes were rinsed with buffer prior to suspension, contamination with other subcellular fractions was minimal if any (Yu 1995). Among the 3 enzymes, activity was highest for JH III epoxide hydrolase and lowest for JH III esterase. Metabolism of JH I by these enzyme systems was found in microsomes prepared from the house fly (*Musca domestica*), the flesh fly (*Sarcophaga bullata*), and the black blow fly (*Phormia regina*) (Yu and Terriere 1978). However, unlike fall armyworm microsomes (toward JH III), JH I esterase activity was 3- to 6-fold higher than JH III epoxide hydrolase activity in different strains of the house fly.

The involvement of a microsomal oxidase system in the metabolism of JH III is supported by the fact that the enzyme is membrane bound, requires NADPH, and is inhibited by the known inhibitors of cytochrome P450, carbon monoxide and piperonyl butoxide. Like JH III, JH I was also reported to be metabolized by microsomal oxidase in insects. Ajami and Riddiford (1973) indicated that microsomal oxidation was involved in JH I metabolism in *Drosophila melanogaster, Thermobia domestica*, and *Hyalophora cercropia*. In agreement with these findings, Yu and Terriere (1978) showed that house fly microsomes prepared from larvae, pupae, or adults possessed microsomal oxidase which matabolized JH I. The oxidase activity in 2 insecticide-resistant strains was much higher than in 2 susceptible strains. JH I oxidase was also detected in *S. bullata* and *P. regina*. Moreover, Hammock et al. (1977) observed NADPH-dependent JH I metabolism by microsomes prepared from insecticide-resistant strains of the house fly. Thus, microsomal oxidases are able to metabolize JH I and JH III in insects. However, the occurrence of JH metabolism by microsomal oxidases in other species is not clear since most in vitro studies have not included the required cofactor, NADPH (Lanzrein and Hammock 1995, Lassiter et al. 1995). Furthermore, the oxidative metabolite of JH, JH diepoxide, which is unstable after oxidation, is converted to tetrahydrofuran (THF) diols via epoxide hydration followed by cyclization (Hammock 1985). THF diols tend to migrate to the position occupied by JH diol in TLC (Yu and Terriere 1978), making definitive conclusions difficult. Perhaps this may explain why oxidative metabolism of JH is not regarded as a general pathway in insects.

JH III diepoxide has been found in several species of insects. Richard et al. (1989) were the first to establish the biosynthesis of JH III diepoxide in larval ring glands of *D. melanogaster*. Recently, Yin et al. (1995) have demonstrated that the corpus allata of adult black blow flies produce JH III diepoxide, JH III, and methyl farnesoate. Bioassay of JH III diepoxide revealed that it possesses JH activity (Richard et al. 1989, Yin et al. 1995). Thus, induction of JH III oxidase by allelochemicals in insects in the present study would not only tend to reduce the JH III titer but also cause the concomitant synthesis of a new JH, JH III diepoxide.

In a previous report (Yu 1995), it was shown that feeding with indoles (indole-3-carbinol, indole-3-acetonitrile), flavonoids (flavone, β-naphthoflavone), monoterpenes (menthol, menthone, peppermint oil), and a coumarin (xanthotoxin) to fall armyworm larvae stimulated midgut microsomal ecdysone 20-monooxygenase activity from 28% to 200% as compared with the controls. The results obtained from the present study show that in addition to those allelochemicals, numerous others including methylenedioxyphenyl compounds and a ketohydrocarbon also induce ecdysone 20-monooxygenase activity. Thus, induction of ecdysone 20-monooxygenase by allelochemicals appears to be a common occurrence in fall armyworms. Azadirachtin, a botanical insecticide, is known to affect ecdysteroid and juvenile hormone titers through blockage of morphogenetic peptide hormone release (e.g., prothoracicotropic hormone; allatotropins) (Mordue and Blackwell 1993). According to Kubo and Klocke (1986), the EC_{50} value (effective concentration causing 50%

growth inhibition) of azadirachtin was 0.4 ppm for fall armyworm larvae. The results of the present study show that this allelochemical had no effect on the biosynthesis of 20-hydroxyecdysone from ecdysone in the fall armyworm. However, azadirachtin was found to inhibit ecdysone 20-monooxygenase activity in vitro in insects (Smith and Mitchell 1988), which may explain its lack of induction of this enzyme.

In the present study, the 4 methylenedioxyphenyl compounds were found to be inducers of ecdysone 20-monooxygenase activity, although these compounds are known inhibitors of microsomal aldrin epoxidase activity in this insect (Yu and Hsu 1993). These results further support the notion that ecdysone 20-monooxygenase differs from xenobiotic-metabolizing cytochrome P450 monooxygenases in the fall armyworm (Yu and Hsu 1993).

It is clear from this study that JH III oxidase and ecdysone 20-monooxygenase can be induced by various allelochemicals in insects. Because levels of juvenile hormone and ecdysone are under rigid endogenous control during insect postembryonic development (Hammock 1985, Smith 1985), changes in their titers due to allelochemical stimulation of these enzymes may cause detrimental effects on the growth and development of phytophagous insects.

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相剋物質誘導草地黏蟲微粒體代謝激素單加氧酶的活性

游錫堅[1]

青春激素 III (JH III) 經由來自草地黏蟲中腸內的微粒體所氧化，這種氧化未連結於微粒體膜上，需要 NADPH 來活化，然而會被一氧化碳和 piperonyl butoxide 所抑制，因此是一典型的微粒體加氧化酶，又稱為 JH III 氧化酶。以吲哚甲醇、荼酚黃酮等相剋物質餵食草地黏蟲的幼蟲，分別提高了 JH III 氧化酶的活性達 188% 和 105% 。這個研究同時利用許多種類的相剋物質，誘導蛻皮素 20–單加氧梅的活性。結果發現許多食物中的成份如多種單萜類、倍半烯萜類、吲哚類等物質可以提高蛻皮素 20–單加氧酶的活性 53%~234% 。其中吲哚類的 harmine 是最強的誘導劑。

關鍵詞：青春激素，蛻皮激素，細胞色素 P450 ，草地黏蟲，相剋物質。

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