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Metabolism of ingested and injected 20-hydroxyecdysone in *Plodia interpunctella* larvae (Lepidoptera, Pyralidae)

Kacem Rharrabe[1,2], Annick Maria[2], Fouad Sayah[1(], René Lafont[2]

[1]*CEEM - Laboratoire de Biologie Appliquée & Sciences de l'Environnement, Faculté des Sciences et Techniques, BP 416, Tangier, MOROCCO.* (*Dead on July 5th, 2008. Corresponding author: Phone +21266430131 Fax: +21239393953 k_rharrabe@yahoo.fr*

[2]*Université Pierre et Marie Curie, Laboratoire Protéines: Biochimie Structurale et Fonctionnelle, CNRS FRE 2852, 7 Quai St. Bernard, 75252 Paris 05, FRANCE.*

Abstract

The metabolic pathways of ingested and injected 20-hydroxyecdysone have been investigated in the fourth instar larvae of *Plodia interpunctella*. *P. interpunctella* larvae display the same qualitative metabolites after ingestion or injection of [^{3}H]20-hydroxyecdysone. In *P. interpunctella*, the detoxification mechanisms are complex, as they involve both dehydrogenation/epimerisation at C-3 and acylation at C-22. We also observed the formation of polar metabolites (20-hydroxyecdysonoic acid and 3-dehydro- (or 3-epi) 20-hydroxyecdysonoic acid). Using a diet supplemented with 100 ppm 20E, we showed that apolar conjugation represents the most efficient pathway of 20E metabolism in *P. interpunctella* larvae.

Keywords: *Plodia interpunctella*, 20-hydroxyecdysone, Ecdysteroid metabolism, detoxification.

Introduction

Ecdysteroids represent a family of steroids that are common amongst invertebrates and plants. In arthropods, it is well established that they are hormones which control development and reproduction (Koolman, 1989). In plants they are generally considered as secondary metabolites, phytoecdysteroids, which protect plants against phytophagous insects (Lafont 1997, Dinan 2001). The major phytoecdysteroid is 20-hydroxyecdysone which is the same molecule as the active hormone in insects. Several studies reported that exogenous application of ecdysteroids in insects provoked a marked growth and developmental disruption by endocrine disrupting system leading to death in these insect species. This was demonstrated in *Pectinophora gossypiella* (Kubo *et al.*, 1981), *Spodoptera frugiperda* (Kubo *et al.*, 1983), *Bombyx mori* (Tanaka & Takeda, 1993), *Agrius convolvulus* (Tanaka & Naya, 1995); *Spodoptera frugiperda*, *Inachis io* and *Aglais urticae* (Blackford & Dinan, 1997), *Lobesia botrana* (Mondy *et al.*, 1997), and in *Bradysia impatiens* (Schmelz *et al.*, 2002).

However, certain species remain unaffected by dietary ecdysteroids. For example, *Helicoverpa virescens* and *H. armigera* would appear to have effective detoxification mechanisms for ingested phytoecdysteroids and are able to consume diets containing 1000 ppm 20E (Kubo *et al.*, 1987) or up to 50 µg ecdysone per insect (Robinson *et al.*, 1987), respectively without any adverse effects on growth and development. This was also observed in *Spodoptera littoralis* (Blackford *et al.*, 1996) and *Lacanobia oleraceae* (Blackford & Dinan, 1997a).

Resistant insects have developed effective detoxification mechanisms against ingested phytoecdysteroids. Generally, these mechanisms proceed mainly though oxidation at C-26, oxidation/epimerization at C-3, or conjugation reactions concerning secondary alcohols (at C-2, C-3 and C-22), which lead to the formation of various polar or apolar inactive conjugates (Lafont & Connat 1989, Rees 1995, Lafont *et al.* 2005). These detoxification mechanism reactions present great variation, depending on the insect species (Rharrabe *et al.*, 2007).

Plodia interpunctella is moderately resistant to ingested ecdysteroids (unpublished data). The aim of the present study was to analyze the metabolic fate of ingested 20-hydroxyecdysone (20E) in fourth instar larvae of *Plodia interpunctella*, and to compare it with that of the injected molecule.

Material and methods

Insects

Plodia interpunctella used in this research were taken as larvae from Errachidia province in South-East region of Morocco. The insects were reared in the laboratory under standard conditions at 28 ± 2°C with a relative humidity of 70 ± 5 % and a photoperiod of 16 : 8 (L:D). Larvae were fed date fruits. Fourth instar larvae were used in this study.

Chemicals

1α,2α-[$^3H_2$]-20-hydroxyecdysone (20E, 40 Ci/mmol) was prepared in the laboratory from labeled custom-made [^{3}H]-2-deoxy-20-hydoxyecdysone and used for metabolic studies.

Standards ecdysteroids (20E, 3-epi-20E, 3-dehydro-20E and 22-fatty acyl esters of 20E) were synthesized or isolated in the laboratory in previous studies.

Treatments

Feeding experiments

Tritiated 20E dissolved in 10 µl of ethanol was incorporated into the diet of *P. interpunctella* larvae which were previously starved during 24 h in order to induce a high feeding rate. Ethanol was allowed to evaporate at 35 °C in an oven. Larvae were left 3 h on treated diet then transferred in other Petri-dishes containing unlabeled diet, and their faeces were collected over the next 24 h. Some experiments were performed using a diet containing 100 ppm of unlabeled 20E in addition to labelled 20E.

Injection experiments

Tritiated 20E dissolved in methanol/ultra pure water (1:9). A volume of 1 µl was injected to each larva (at abdominal level) using a 10 µl Hamilton syringe. Before being injected, the larvae were anesthetized in the cold (at 4ºC for 2 min). They were left individually in Petri-dishes containing untreated diet, and their faeces were collected over the next 24 h.

Extractions of excreta

Faeces were extracted with 1mL methanol using a sonication bath during 30 min, then centrifuged at 9,000 rpm for 5 min. The solvent was dried under nitrogen at 25°C. Dried samples were redisolved in 1 ml of methanol. Aliquotes (10µl) were mixed with liquid scintillation cocktail (3ml) then assayed for radioactivity by using a Kontron Beta IV liquid scintillation counter. Then, samples were analyzed by radio-HPLC with different chromatographic systems.

Identification of ecdysteroids

Chromatographic procedures

Chromatographic analysis of radioactive metabolites was performed by RP-HPLC using a Thermoseparation apparatus with a variable UV detector set at 245 nm. Flow-rate was 1 mL/min in every case. Three different chromatographic systems were used:

- Chromatographic system 1: RP-HPLC used an ACE 5C18-HL column (150 x 4.6 mm i.d.) eluted with 17% acetonitrile-isopropanol (5:2) in 20mM Tris-$HClO_4$, pH 7.5 during 20 min, then 100% acetonitrile-isopropanol (5:2) for 15 min.

- Chromatographic system 2: same as system 1, except that Tris was replaced by 0.1% trifluoroacetic acid (TFA) in water.

- Chromatographic system 3: RP-HPLC was performed by using a Spherisorb 5ODS2 column (250 x 4.6 mm i.d.) and a gradient of acetonitrile-isopropanol (5:2 v/v) in 20 mM Tris/$HClO_4$, pH 7.5 (8 to 40% in 40 min, then 40 to 100% in 10 min, then 100% of acetonitrile-isopropanol for 20 min).

Radioactivity in the effluent was analyzed with in-line radioactivity monitor (Flo-One A 250, Packard) equipped with a 0.5 mL detection cell. The scintillation cocktail was Ecoscint™ used at a flow-rate of 3 mL/min. The identification of the various labelled metabolites present in the samples was based on their co-migration with reference ecdysteroids using two different chromatographic systems.

Enzymatic hydrolyses

The characterization of polar or apolar conjugates was performed by enzymatic hydrolysis using two different systems.

Polar metabolites: aliquotes of each extract were evaporated and dissolved in 1 mL of 50 mM sodium acetate buffer, pH 5.4, and incubated overnight with 1 mg ß-glucuronidase from *Helix pomatia* (H1 type, Sigma) at 37°C.

Apolar metabolites: samples were evaporated and dissolved in 1 mL of 20 mM borate buffer, pH 8.5, and incubated overnight with 1 mg porcine liver esterase (Sigma) at 37°C.

In both cases, ecdysteroids were then adsorbed on a C18 Sep-Pak cartridge (Millipore) and eluted with 5 ml of methanol.

Results

Metabolism of ingested 20-hydroxyecdysone in *P. interpunctella* larvae

Ingested radiolabelled 20E was transformed into three sets of compounds of decreasing polarity (Figure 1): the first group was polar, the second exhibited a polarity close to that of 20E, and the third group corresponded to apolar metabolites. The first two groups were easily separated from the third one using a C18 Sep-Pak cartridge (Millipore) which was eluted with 5 ml of 60% methanol (SP60) and then with 5 ml of 100% methanol (SP100). The SP60 and the SP100 (the latter after esterase hydrolysis) were analyzed by HPLC system 2 (Figure 1). Such conditions allowed to fully characterize the different peaks. Metabolites of polarity close to 20E coeluted with reference 3-epi-20-hydroxyecdysone (3-epi-20E) and 3-dehydro-20-hydroxyecdysone (3D20E), respectively (3D20E gives two peaks under these conditions). The two polar peaks were ionizable, their retention time varied with solvent pH (it increased at acidic pH), and they remained unchanged after enzymatic hydrolyses (data not shown). So, these metabolites are not conjugates and their behaviour indicates that the more polar is probably 20-hydroxyecdysonoic acid (20Eoic) and the second either 3-dehydro-20-hydroxyecdysonoic acid or 3-epi-20-hydroxyecdysonoic acid. The SP100 fraction after hydrolysis gave 20E, 3-epi-20E and 3D20E. So the apolar metabolites correspond to conjugates of 20E (and to a lower extent of 3-epi-20E and 3D20E) with various fatty acids (most probably 22-acyl esters as in other insect species). There were no apolar esters of ecdysonoic acids.

Metabolism of ingested surcharged 20-hydroxyecdysone in *P. interpunctella* larvae

Faeces of larvae having ingested a diet labelled with [^{3}H]-20E and surcharged with 100 ppm of unlabeled 20E presented a simplified chromatographic profile as compared with the previous one. The majority of the radioactivity was present as 20E and apolar conjugates of 20E with various fatty acids (Figure 2A). Thus apolar conjugation represents the most effective and least saturable inactivation mechanism of 20E metabolism in *P. interpunctella* larvae.

Metabolism of injected 20-hydroxyecdysone in *P. interpunctella* larvae

Injected [^{3}H]20E into larvae follows the same fate as the ingested one. Radiolabelled 20E was transformed into the same three groups of compounds

(Figure 2B) as observed during ingestion experiments. However, the proportions of the different metabolites were very different between injection and ingestion experiments. Indeed, metabolites of group I were highly increased [31.5% (injection experiment) vs. 10% (ingestion experiment) of the total radioactivity] and metabolites of group III were decreased noticeably [50% (injection experiment) vs. 72% (ingestion experiment)].

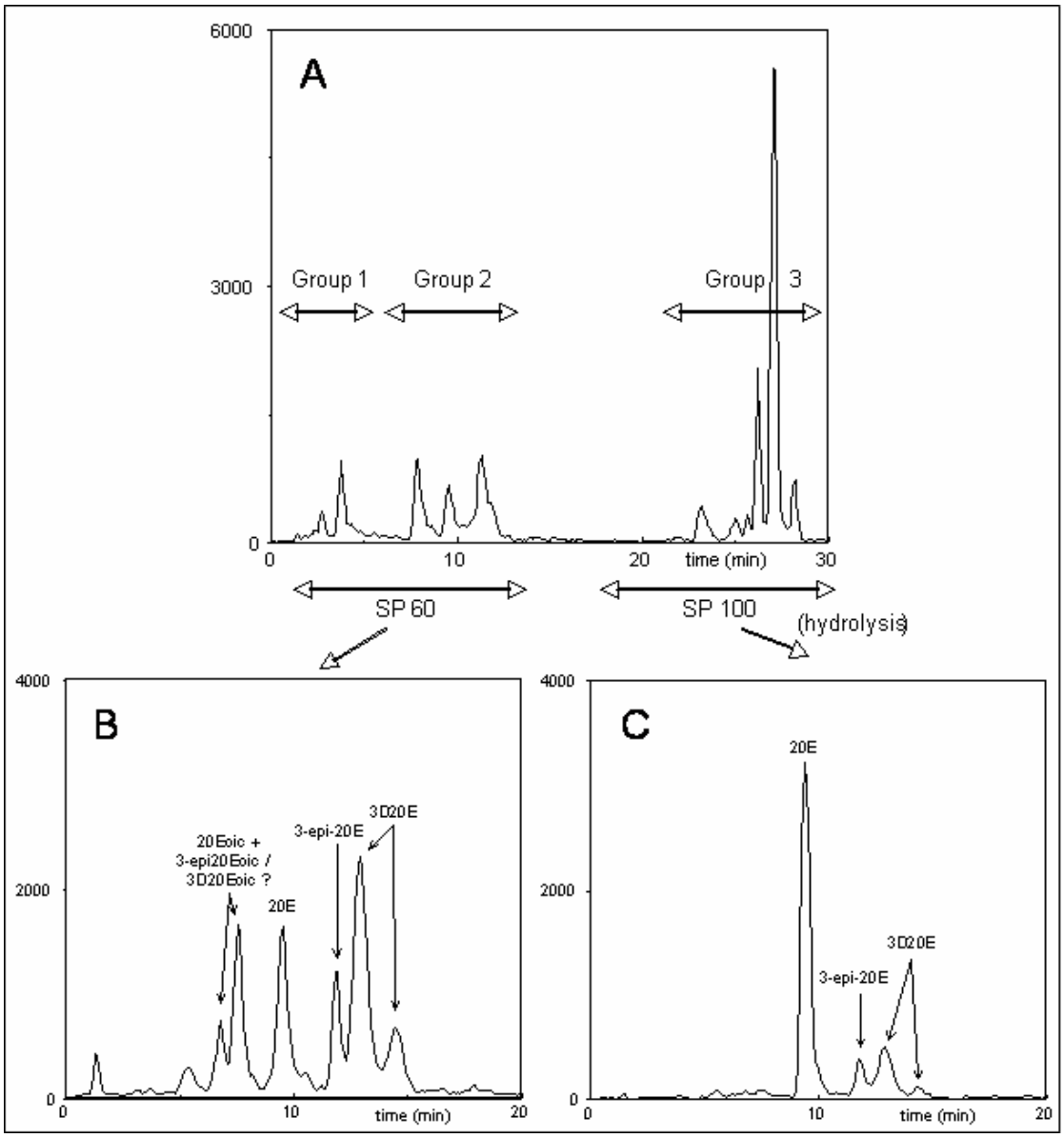

Figure 1. RP-HPLC analysis of radioactive metabolites in faeces of fourth instar larvae of *Plodia interpunctella* following ingestion of a diet containing [^{3}H]-20E.
A: Analysis of an aliquote of the whole extract (chromatographic system 1).
B: Analysis of fraction SP60 (chromatographic system 2).
C: Analysis of fraction SP100 after esterase treatment (chromatographic system 2).

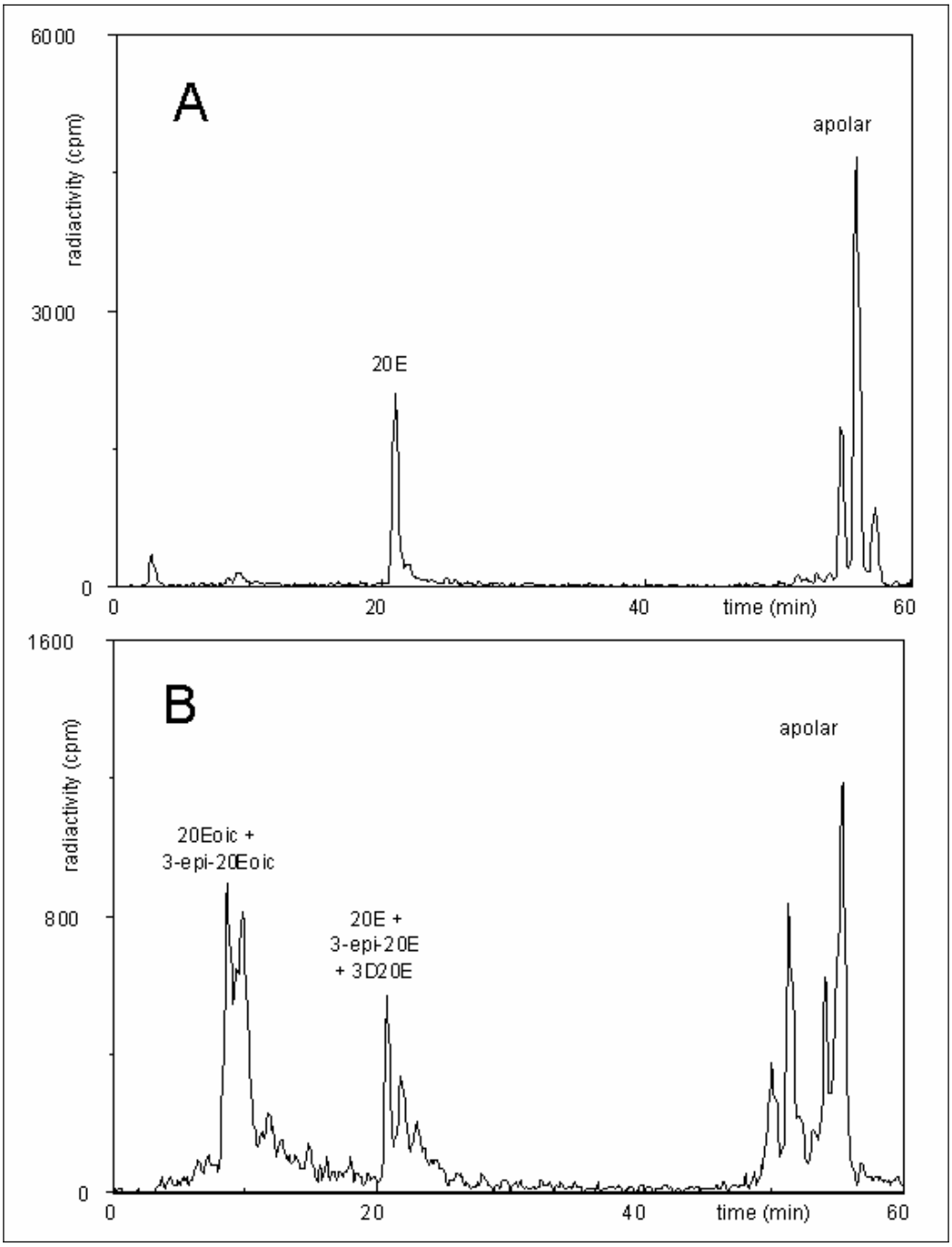

Figure 2. RP-HPLC analysis of radioactive metabolites in faeces of fourth instar larvae of *Plodia interpunctella* (chromatographic system 3). A: after ingestion of a diet treated with [^{3}H]-20E and 100 ppm 20E. B: after injection of [^{3}H]-20E.

Discussion

The metabolic pathways of ecdysteroids present a great diversity among insect species (Rharrabe *et al.*, 2007). A common pathway is represented by the formation of ecdysonoic acids but diversity concerns the secondary hydroxyl groups at C-2, C-3 and C-22, which can be conjugated with either polar or apolar moieties (Lafont *et al.*,

2005). In addition, the 3-OH can undergo oxidation and epimerization. The nature of the detoxification mechanisms shows no correlation with insect taxonomy, and Lepidopteran, which represent the most studied order, use very different pathways (Hikino *et al.* 1975, Beydon *et al.* 1987, Kubo *et al.* 1987, Robinson *et al.* 1987, Blackford *et al.* 1996, Blackford & Dinan, 1997a, Rharrabe *et al.* 2007). Among Lepidoptera, some species (*Heliothis virescens* - Zhang & Kubo, 1993; *Heliothis armigera* - Robinson *et al.*, 1987; *Ostrinia nubilalis* - Rharrabe *et al.*, 2007) produce 22-fatty acyl esters, whereas other ones (*Manduca sexta* - Weirich *et al.*, 1991; *Pieris brassicae* - Beydon *et al.*, 1987; *Spodoptera littoralis* - Webb *et al.*, 1996) produce mainly 3-epimers, and *Bombyx mori* cleaves the side-chain between C-20 and C-22 (Hikino *et al.*, 1975). In addition, the 3-epimers are converted into 3-phosphates in *Pieris brassicae* (Beydon et al., 1987) and into 3-sulfates in *Bombyx mori* (Zhang & Kubo, 1993), whereas *Spodoptera littoralis* produces 2-phophates (together with small amounts of 22-phosphates) of both 3β-OH, 3-oxo and 3α-OH ecdysteroids (Webb *et al.*, 1995). With the exception of 26-oxidation, which is active in all tissues (and is used to inactivate endogenously produced ecdysteroids), all these reactions take place in insect gut and are expected to provide a more or less efficient protection against ingested phytoecdysteroids.

The formation of 3-epimers is a two-step cytoplasmic process (Weirich & Svoboda, 1992) which takes places exclusively in the gut of some Lepidoptera during the feeding larval instars and disappears during metamorphosis. The formation of apolar conjugates was widely reported in insects. Zhang & Kubo suggested that the main role of the 22-acyltransferase (present in the plasma membrane of the gut epithelial cells) is related to detoxify ingested molecules such as phytoecdysteroids (Zhang & Kubo, 1992, 1994). This acyltransferase is active only during feeding stage, and its activity decreases as the larvae become committed to pupation. In the fifth instar larvae of *H. virescens*, its activity is not increased by adding ecdysteroids to the larval diet (Zhang & Kubo, 1992).

P. interpunctella displays a complex "hybrid" pattern, as it involves both epimerization at C-3 and conjugation with fatty acids (expected to take place at C-22, as in all other species studied so far). However this species is not so resistant to ingested ecdysteroids, and deleterious effects are observed when the diet contains more than 200 ppm of 20E (unpublished data). Indeed, the fact that the intestinal barrier here is not 100% effective and that some ingested 20E crosses the gut wall is suggested by the presence of significant amounts of 20-hydroxyecdysonoic acid in the larval faeces (Figure 1). A more efficient gut barrier was found in other species *Heliothis armigera* (Robinson *et al.*, 1987) and *Ostrinia nubilalis* (Rharrabe *et al.*, 2007), as no polar metabolites (and no 20E) were found in faeces after ecdysone ingestion. However, we are aware of the limits of our argument, which does not fit with the results of figure 2A, as in this case almost no ecdysonoic acids were observed, although it is clear that some 20E must have been absorbed, in order to evoke the observed developmental disorders (unpublished data). Additional experiments will hopefully help us to solve this paradox.

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