Biological Activity of Some Moulting Hormone Agonists in Mealworms: Ecdysteroid and Protein Analysis in Ovaries

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ABSTRACT

In a topical (10 µg/female) bioassay, three moulting hormone agonists (RH-0345, RH-5849, RH-5992) were tested on newly emerged adult females of the mealworm, *Tenebrio molitor* (Coleoptera: Tenebrionidae), under *in vivo* conditions. In a first series of experiments the compounds were assayed on ovarian ecdysteroid production measured at day 2 following treatment, corresponding to the beginning of vitellogenesis. Data from enzyme immunoassay measurements revealed that all tested compounds resulted in a significant increase in ovarian ecdysteroid amounts. Moreover, RH-5992 was found more active than RH-0345 and RH-5849. In a second series of experiments, the activity of these compounds was investigated on ovarian proteins. The ecdysteroid agonist RH-5849 caused a significant reduction of protein amounts in the ovaries. The electrophoretic separation of ovarian extracts from treated series showed that one or two protein bands were missing compared to controls. These observations may suggested an interference of ecdysteroid agonists with the vitellogenesis process.

KEY WORDS: Mealworm, Insecticides, Ovaries, Hormones, Ecdysteroids, Proteins

Résumé

Trois mimétiques de l'hormone de mue (RH-0345, RH-5849, RH-5992) ont été testés par application topique (10 µg/femelle) sur les adultes femelles de *Tenebrio molitor* (Coleoptera: Tenebrionidae), en conditions *in vivo*. Dans une première série d'expériences, les composés ont été évalués sur la production d'ecdystéroïdes ovariens mesurée à l'âge deux jours après traitement correspondant au début du processus de la vitellogénèse. Les résultats des dosages enzymo-immunologiques des hormones révèlent que tous les produits testés provoquent une augmentation significative des taux ovariens d'ecdystéroïdes. De plus, RH-5992 et RH-0345 sont plus actifs que RH-5849. Dans une seconde expérimentation, l'effet de ces composés a été examiné sur les protéines ovariennes. Seul le RH-5849 réduit significativement le taux de protéines dans les ovaires. La séparation électrophorètique des extraits ovariens montre l'absence d'une ou de deux fractions protéiques chez les séries traitées comparativement aux témoins. Ces observations suggèrent une interférence des agonistes des ecdystéroïdes avec le processus de vitellogénèse.

Mots clés : *Tenebrio molitor*, Insecticides, Ovaires, Hormones, Ecdystéroïdes, Protéines

Introduction

Dibenzoylhydrazine derivatives are nonsteroidal ecdysteroid agonists that mimic the action of the moulting hormones (ecdysteroids) and induced a precocious and incomplete moult in several insect orders (Wing, 1988; Smagghe and Degghele, 1994; Oberlander *et al.*, 1995; Retnakaran *et al.*, 1995; Dhadialla *et al.*, 1998). Recently, we have found that treatment of RH-0345 in pupae of the mealworm *Tenebrio molitor* could increase both the hemolymph ecdysteroid peak and the amount of ecdysteroid released by sternal explants into the culture medium, and in addition a new cuticle was secreted in a manner similar to 20-hydroxyecdysone (Soltani *et al.*, 2002). In addition, RH-0345 was found to affect growth and development of ovaries (Soltani *et al.*, 1998), and increased the ovarian ecdysteroid production in *T.*

molitor (Taïbi *et al.*, 2003). In order to extend these findings, the present study was designed to compare the biological activity of three ecdysteroid agonists (RH-5849, RH-5992, RH-0345) applied topically on newly emerged adult females of *T. molitor* an important pest in stored products world wide. Firstly, the ecdysteroid agonists were assayed on ovarian ecdysteroid production recorded at the beginning of the vitellogenesis phase. Then, their effects were also tested on both the amount and pattern of ovarian proteins since the follicular cells are know as the site of biosynthesis of ecdysteroids and proteins (Rees, 1995; Gäde *et al.*, 1997).

Materials and methods

Insects. *T. molitor* pupae were removed daily from a stock colony, sexed according to Bhattacharaya *et al.* (1970) and kept separately until adult emergence. Adults were collected at 0-4 h following emergence and reared on wheat flour at 27°C and 80% R.H. in almost continuous darkness.

Insecticides and treatment. Technical RH-5849, RH-5992 and RH-0345 were kindly supplied by Rohm and Haas Co. (Spring House, PA, USA). The compounds were dissolved in acetone and topically applied in 3 µl of acetone to newly emerged adult females (10 µg/female). Control insects were treated with acetone alone (3 µl per female).

Enzyme immunoassay for measurement of ecdysteroids. At appropriate times, ovaries were collected and ecdsyteroids extracted (Soltani-Mazouni *et al.*, 1999). Individual sample was analyzed in duplicate by an enzyme immunoassay (EIA) of Porcheron *et al.* (1989) as previously described (Soltani *et al.*, 2002) using a conjugate of 20-hydroxyecdysone coupled to peroxidase as enzymatic tracer, tetramethyl benzidine as a color reagent and a rabbit B polyclonal antibody. Data are expressed as pg ecdysone equivalents/mg ovaries.

Determination of ovarian protein amounts. Ovaries were dissected from control and treated females at appropriate times during the adult life, weighed and subjected to protein extraction (Soltani *et al.*, 1996). Each pair of ovaries was homogenized in 1 ml of trichloracetic acid (20%) and centrifuged (5,000 g for 10 min). Protein content of ovaries was estimated by the Coomassie Blue method of Bradford (1976) using BSA as a standard.

Electrophoresis. Ovaries were collected from 2-days old females in control and treated series and subjected to protein extraction (Soltani *et al.*, 1996). The protein extracts from pooled sample (8-10 paired ovaries per series) were analyzed on 8% sodium dodecyl sulfate-polyacrylamide slab gels (SDS-PAGE) following the procedures of Laemmli (1970). Gels were stained with Coomassie Blue Brillant R 250 (Merck) and molecular weights were estimated with a set of protein markers.

Statistical analysis. Results are expressed as means±s, and comparison of mean values between control and treated series was estimated by Student's t -test at 5% level.

Results

Effects on ovarian ecdysteroid production. Under normal conditions, the amount of ecdysteroids produced *in vivo* by ovaries during the sexual maturation increased until oviposition (day 4) and declined thereafter (Soltani-Mazouni *et al.*, 1999). The ecdysteroid agonists were applied topically at 10 µg/individual on newly emerged adult females and ovarian ecdysteroid production was measured at day 2 following treatment corresponding to the beginning of vitellogenesis. Table 1 demonstrates a significant ($p< 0.05$) increase in the ovarian ecdysteroids amounts measured at day 2 after treatment with all three compounds compared to controls. The most active was RH-5992, followed by RH-0345 and then RH-5849.

Table 1. Effect of ecdysteroid agonists applied topically on newly emerged adult females of *Tenebrio molitor* on the amount of ecdysteroids (pg ecdysone equivalents/mg) in ovaries. Values followed by the same letter are not significantly different at $p< 0.05$ from the control (m±s, n=4 females).

Age (days)	Control	RH-0345	RH-5849	RH-5992
2	33.8 ± 4.5 a	70.8 ± 9.4 b	61.2 ± 4.4 b	95.3 ± 5.3 c

Effects on ovarian protein. At 2 days after topical treatment of newly emerged adult females, the effect on ovarian protein amounts was only significant for RH-5849 (Table 2). At this moment of day 2, females are at the beginning of the vitellogenesis phase.

Table 2. Effect of ecdysteroid agonists applied topically on newly emerged adult females of *Tenebrio molitor* on ovarian protein amounts (µg/mg tissue) recorded at 2 days following treatment. Values followed by the same letter are not significantly different at $p< 0.05$ from the control (m±s, n=4 females).

Age (days)	Control	RH-0345	RH-5849	RH-5992
2	9.8 ± 9.2 a	08.1 ± 6.4 a	8.4 ± 9.8 b	118.2 ± 8.2 a

Females were treated at days 0 and ovaries dissected at day 2. Extracted ovarian proteins were analysed by SDS-PAGE and electrophoretic patterns were shown in figure 1. In controls, we could detect 8 protein bands in the ovarian protein pattern with molecular weights varying approximatly from 56 to 145 kDa. Only two bands presented a molecular weight higher than 116 kDa. Treatments with the ecdysteroid agonists resulted in a slight reduction in the number and the intensity of some protein bands compared to controls. The molecular weights were determined with a set of protein markers. More specifically, it was possible to detect in the patterns of ovarian proteins from 2-days old treated females the absence of one band, namely a band of 96 kDa, with RH-0345 and RH- 5992 or two bands of 77 and 96 kDa with RH-5849, respectively (Figure 1).

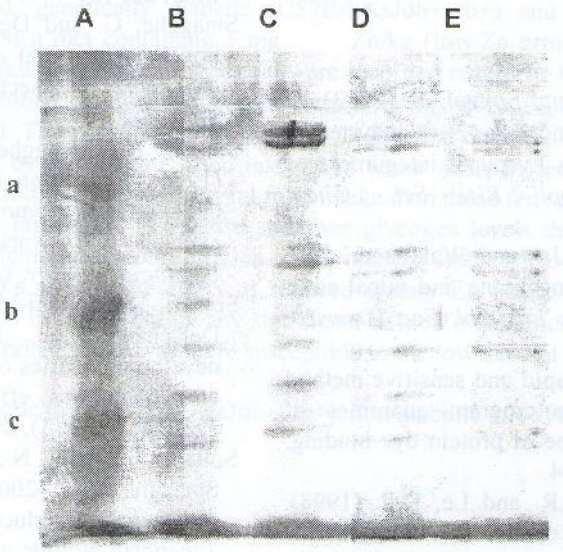

Figure 1. Electrophoretic separation by SDS-PAGE (8 %) of ovarian proteins in control and treated series from 2-days old females after topical application on newly emerged adults of *Tenebrio molitor*. (**A**) Protein markers (**a**, B-galactosidase, 116 kDa; **b**, phosphorylase, 97 kDa; **c**, albumin, 66 kDa); (**B**) Control; (**C**) RH-0345; (**D**) RH-5992; (**E**) RH-5849.

Discussion

The first two ecdysteroid agonists reported, RH-5849 and RH-5992, appear to be highly active against Lepidoptera, while RH-0345 presents a high activity in Coleoptera (Dhadialla et al., 1998). In mealworm adults, RH-0345 was found to reduce both growth and development of oocytes in vitro (Soltani et al., 1998) and the thickness of the follicular epithelium in vivo (Taïbi et al., 2003). When tested on T. molitor pupae, RH-0345 appeared to be the most potent in the hormonal release by pupal integuments as compared with the two lepidopteran-selective ecdysteroid agonists RH-5849 and RH-5992 (Soltani et al., 2002). In addition, EIA measurements of ecdysteroids released into the culture medium by pupal integumental explants, showed that RH-0345, either alone or followed by KK-42, an imidazole derivative antagonist of moulting hormone, resulted in higher amounts of ecdysteroids as compared to controls (Berghiche et al., 2003). Similarly, the present study conducted under in vivo conditions, showed that the tested ecdysteroid agonists increased the ovarian ecdysteroid amounts confirming previous results (Lorenz et al., 1995; Soltani et al., 2002). In addition, RH-5992 and RH-0345 were more active towards the ecdysteroid production by ovaries than RH-5849. According to Williams et al. (2002), the high toxicity of RH-5992 and RH-0345 in Lepidoptera might be correlated to a greater induction of 26-hydroxylase, an ecdysteroid inactivation enzyme.

It is a general phenomenon that the follicular cells produce ecdysteroids (Rees, 1995; Gäde et al., 1997) and proteins (Raikhel and Dhadialla, 1992). In the current study, it was demonstrated that the three ecdysteroid agonists affected the ovarian proteins as evidenced by protein amounts and SDS-PAGE. The effects observed on the protein patterns varied as function of the compound. Indeed, one or two protein

bands with molecular weights of 77 and 96 kDa were missing compared to controls. Similarly, Lawrence (1992) found a reduction of protein synthesis and/or incorporation into eggs due to RH-5849 in Anastrepha suspensa. Farinos et al. (1999) also demonstrated clear negative effects of RH-0345 in yolk protein accumulation and egg formation in Leptinotarsa decemelineata. All these and other observations obtained so far suggest an interference of ecdysteroid agonists with the vitellogenesis process in mealworms. Further investigations are needed to give more details on the action site(s).

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