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Stimulation of Juvenile Hormone Biosynthesis by Different Ecdysteroids in *Bombyx mori*

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Shi-Hong Gu and Yien-Shing Chow (2003) Stimulation of juvenile hormone biosynthesis by different ecdysteroids in *Bombyx mori*. *Zoological Studies* **42**(3): 450-454. Precisely controlled juvenile hormone (JH) biosynthesis by the corpora allata (CA) is critical for insect postembryonic development. In the present study, the effects of different ecdysteroids (20-hydroxyecdysone, ecdysone, 2-deoxy-20-hydroxy-ecdysone and 20-hydroxyecdysone-22-acetate) on both the induction of supernumerary larvae and CA activity in vitro were further clarified. We found that 20-hydroxyecdysone as well as ecdysone and 2-deoxy-20-hydroxy-ecdysone stimulated CA activity both in vivo and in vitro. However, 20-hydroxyecdysone-22-acetate had no effect on CA activity. The direct activation of in vitro JH biosynthesis by 20-hydroxyecdysone, ecdysone, and 2-deoxy-20-hydroxyecdysone indicates that different ecdysteroids may exert similar in situ effects in a coordinated manner for eliciting specific events during development. We propose that the activation of JH biosynthesis by elevated hemolymph ecdysteroid levels in larvae treated with different ecdysteroids may be directly responsible for the induction of supernumerary larval molting. http://www.sinica.edu.tw/zool/zoolstud/42.3/450.pdf

Key words: Ecdysteroid specificity, Supernumerary larvae, In vitro CA activity, Postembryonic development, *Bombyx mori*.

Insect molting and metamorphosis are regulated by 2 classes of hormones: ecdysteroids and juvenile hormones (JH) (Gilbert et al. 1996 2002). Ecdysteroids are a family of polyhydroxylated steroids that are the molting hormones. In larvae of most insect species, the prothoracic glands synthesize and secrete ecdysone, which is hydroxylated by peripheral tissues to form 20-hydroxyecdysone. 20-Hydroxyecdysone then acts on target tissues such as the epidermis to elicit hormonal effects (Smith 1985, Gilbert et al. 1996 2002). JH is a sesquiterpene that is synthesized and secreted by the corpora allata (Riddiford 1994, Yin 1994, Ho et al. 1995, Kou and Tu 1998, Kou and Chen 2000). It is generally accepted that during insect postembryonic development, the interplay of ecdysteroids and JH serves to orchestrate the progression from one developmental stage to the next, with ecdysteroids initiating the molting process and JH regulating the quality of the molt. Numerous studies have been conducted to clarify the developmental regulation of these 2 important hormones (Smith 1985, Gilbert et al. 1996 2002, Gu and Chow 2001 2003).

With the introduction of an ecdysteroid radioimmunoassay (Borst and O'Connor 1972), precisely controlled fluctuations in ecdysteroid titers during development have been determined in various insect species (Smith 1985, Sehnal 1989, Gilbert et al. 1996 2002). In the larvae of the silkworm, *Bombyx mori*, it has been demonstrated that a difference exists in the baseline hemolymph ecdysteroid levels between the early 4th and 5th (last) larval instars: low but significant levels (30-40 ng/ml) are observed during the early 4th instar (Calvez et al. 1976, Kiguchi and Agui 1978), and these become very low during the early stages of the last instar (Calvez et al. 1976). Previously we demonstrated that this difference in baseline ecdysteroid levels has an important physiological significance for larval-pupal transformation in this insect, with very low ecdysteroid levels

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during the early last larval instar initiating inactivation of the CA and playing a role in directing metamorphosis (Gu and Chow 1993 1996, Gu et al. 1995a). It was found that when these very low ecdysteroid levels were artificially elevated to levels similar to those in the early stages of the 4th instar by treating the food source with 20-hydroxyecdysone, a decline in JH biosynthesis by the corpora allata (CA) was prevented and therefore an additional larval molting occurred (Gu and Chow 1993 1996, Gu et al. 1995a).

In the present study, the specificity of different ecdysteroids for inducing supernumerary larval molting and for stimulating CA activity in vitro was further clarified. We tested 4 ecdysteroids that are commercially available and found that 20-hydroxyecdysone as well as ecdysone and 2-deoxy-20-hydroxyecdysone have a stimulatory effect on JH biosynthetic activity of the CA both in vivo and in vitro.

MATERIALS AND METHODS

Insects

Larvae of the tetramolter silkworm, *B. mori*, were reared on fresh mulberry leaves at 25°C under a 12 L:12 D photoperiod. Newly-ecdysed 5th (last) instar larvae were collected and used for each experiment.

Application of ecdysteroids

20-Hydroecdysone, ecdysone, 2-deoxy-20-hydroxyecdysone, and 20-hydroxyecdysone-22-acetate (Sigma, St. Louis, MO) were dissolved in ethanol as a stock solution at a concentration of 1 mg/ml, diluted in distilled water to several different concentrations (ecdysone: 5, 10, 20, and 100 ppm; 20 ppm for the other ecdysteroids). A total of 100 ml for each concentration was then applied topically to mulberry leaves. Newly-ecdysed last instar larvae were fed on these treated mulberry leaves and development of the larvae was then carefully observed. Control larvae were fed on fresh leaves that were treated with water and ethanol only.

Radiochemical assay of JH synthesis

JH biosynthesis and secretion by the CA were measured in vitro using a radiochemical assay according to procedures described in a previous study (Pratt and Tobe 1974), as modified by others (Gu et al. 1995, Ho et al. 1995b). The rate of in vitro ^{3}H-methionine incorporation by the CA was expressed as pmol/gland pair/h.

RESULTS

Induction of supernumerary larval molting by applying different ecdysteroids to the food source

Our previous studies (Gu and Chow 1993 1996) showed that when last instar *B. mori* larvae were fed with 20-hydroxyecdysone-supplemented mulberry leaves, inactivation of the CA, which normally occurs midway through the last larval instar (Gu and Chow 1996), was prevented, and an addi-

Table 1. Effects of ecdysteroid supplementation of the food source on development of last instar larvae

Type of ecdysteroids	Concentration	Percent of supernumerary larvae
Ecdysone	100 ppm	60%
Ecdysone	20 ppm	100%
Ecdysone	10 ppm	30%
Ecdysone	5 ppm	0%
20-Hydroxyecdysone	20 ppm	100%
2-Deoxy-20-hydroxyecdysone	20 ppm	60%
20-Hydroxyecdysone-22-acetate	20 ppm	0%

Ecdysone, 20-hydroxyecdysone, 2-deoxy-20-hydroxyecdysone, and 20-hydroxyecdysone-22-acetate were dissolved in ethanol as stock solutions, diluted in distilled water to concentrations of 5, 10, 20, or 100 ppm, and then applied topically to mulberry leaves, respectively. Larvae were fed with these treated mulberry leaves throughout the last instar and the development of larvae was carefully observed. Ten animals were used in each treatment.

tional supernumerary larval molting was thus induced at the end of the instar (Gu and Chow 1993 1996). In the present study, the specificity of different ecdysteroids (ecdysone, 20-hydroxyecdysone, 2-deoxy-20-hydroxyecdysone, and 20-hydroxyecdysone-22-acetate) to induce supernumerary larval molting was tested. As shown in table 1, 20-hydroxyecdysone as well as ecdysone and 2-deoxy-20-hydroxyecdysone induced supernumerary larval molting at the end of the instar. The dose-dependent effects of ecdysone treatment showed that the most effective dose was 20 ppm, similar to that of 20-hydroxyecdysone (Gu and Chow 1993). For treatment with 20 ppm of 2-deoxy-20-hydroxyecdysone, only 60% of the larvae underwent an additional larval molting. We also changed the concentrations of both 20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone (100, 10, and 5 ppm) and found that 20 ppm was the most effective dose (Gu, unpubl. data). However, 20-hydroxyecdysone-22-acetate treatment had no such effect.

Stimulation of CA activity by ecdysteroids in vitro

In subsequent experiments, the effect of different ecdysteroids on the in vitro stimulation of CA activity was further clarified. The CA were isolated from newly ecdysed last instar larvae and then incubated in vitro in TC 199 containing different ecdysteroids or control TC 199. After 48 h of incubation, incorporation of ^{3}H-methionine was assayed. As shown in figure 1, 20-hydroxyecdysone, ecdysone, and 2-deoxy-20-hydroxyecdysone had a stimulatory effect on CA activity; much more JH was produced when the medium contained these ecdysteroids as compared to the control medium. By contrast, no stimulation of CA activity was observed when the medium contained 20-hydroxyecdysone-22-acetate, implying that this is an inactive ecdysteroid metabolite. The concentration of ecdysteroids in the medium was chosen to be 25 ng/ml, because our previous study on the dose-dependent effects of 20-hydroxyecdysone showed that 25 ng/ml was most effective for stimulating in vitro JH biosynthesis (Gu and Chow 1996).

DISCUSSION

The present study clearly shows that 20-hydroxyecdysone, as well as ecdysone, 2-deoxy-20-hydroxyecdysone have a stimulatory effect on CA activity both in vivo and in vitro. In vivo, when these ecdysteroids were applied to the food source during the last larval instar, an additional supernumerary larval molting was induced instead of metamorphosis, indicating that these ecdysteroids stimulate CA activity. Incubation of CA with these ecdysteroids in vitro indicated that the action of ecdysteroids on CA activity is direct. However, 20-hydroxyecdysone-22-acetate had no such effect, implying that it is an inactive metabolite. 2-deoxy-20-hydroxyecdysone is considered to be a precursor of 20-hydroxyecdysone (Ree 1989), and although ecdysone may have some hormonal effects *per se*, it appears to function primarily as a precursor of 20-hydroxyecdysone, which is considered to be the true molting hormone (Smith 1985). Thus it is of interest that not only the active form of the molting hormone, but also its precursors had the same effect on CA activity: their presence stimulated the CA to continue producing JH in vitro. It has been demonstrated in several insect species that dietary applications of different ecdysteroids either have little effect on larval growth and development (Robbins et al. 1968) or cause

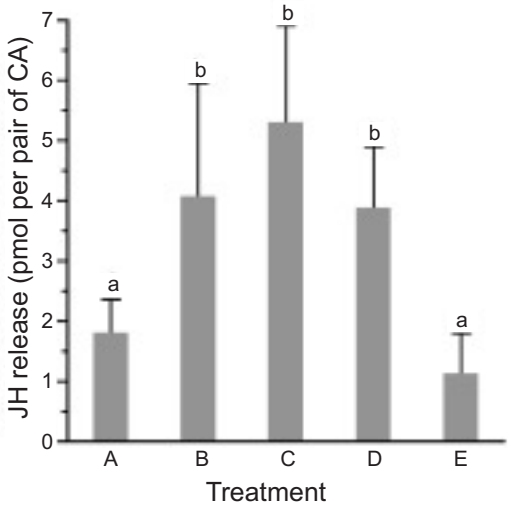

Fig. 1. Cumulative release of JH from the CA of newly-ecdysed last instar larvae. CA were maintained in vitro in the absence (Treatment A) or presence of 20-hydroxyecdysone (Treatment B) or ecdysone (Treatment C), 2-deoxy-20-hydroxyecdysone (Treatment D) or 20-hydroxyecdysone-22-acetate (Treatment E). The concentration of each ecdysteroid in the medium was 25 ng/ml. The CA were maintained in vitro for 48 h. The amount of JH released was determined using a radiochemical assay. Each point represents the mean value from 6 separate assays. Bars indicate the S.E. Dissimilar superscript letters indicate a significant difference (Student's *t*-test, $p < 0.01$).

mortality without an additional larval molting (Singh and Russel 1980, Kubo et al. 1983). It has been reported that in *B. mori*, ecdysone and 20-hydroxyecdysone supplements to the diet affect development of larvae differently, with ecdysone eliciting supernumerary larval ecdysis (Tanaka and Takeda 1993). The discrepancy between our results and others may have been due to differences in developmental stages, in treatment methods, in tissue responses, as well as in insect species.

The same effects on CA activity both in vivo and in vitro by different ecdysteroids presented in this study indicate that different ecdysteroids in situ may exert similar effects in a coordinated manner for eliciting specific events during development. Considering that the ratio of each ecdysteroid may constantly change (Smith 1985, Warren and Gilbert 1986), this result is not surprising. However, to elicit specific developmental events, quantitative differences in the actions among different ecdysteroids exist; for example, in an in vitro system, 20-hydroxyecdysone is one to several hundred times more active than ecdysone in eliciting puffing in *Drosophila melanogaster* (Ashburner 1971). It has been reported that during pupal-adult development of *Manduca sexta*, different ecdysteroid forms exist whose ratios change in tissue-specific development-dependent manners, implying that different ecdysteroids may have specific roles in development (Warren and Gilbert 1986). It is of interest to note that different ecdysteroids may sometimes play separate roles, or may exert the same action in a coordinated manner during other developmental stages. Exploring this complexity can lead to a clearer understanding of how insect systems have survived the long evolutionary period using ecdysteroids as one of their key developmental hormones.

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