Eur. J. Entomol. **92**: 155–160, 1995
ISSN 1210–5759

The different effects of ecdysone and 20-hydroxyecdysone on the induction of larval ecdysis in the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)

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***Bombyx mori*, larval molt, ecdysone, 20-hydroxyecdysone, ecdysteroid level, larval apolysis**

Abstract. The silkworm, *Bombyx mori* was reared on artificial diets supplemented with 20-hydroxyecdysone (20E) during the 4th instar and on diets supplemented with various concentrations of either 20E or ecdysone (E) in the 5th (last) instar. The larvae transferred to diet containing E molted to the 6th instar earlier than the control larvae and their mortality was within norm. On the other hand, most of the larvae that were transferred to the diet supplemented with 300 ppm 20E continued to feed until they died without apolysis. The amount of 20E in their hemolymph was higher than in the larvae fed 200 ppm E. Larvae transferred to a diet containing both E and 20E did enter apolysis and their ecdysteroid level was similar as in the non-molting larvae receiving 300 ppm 20E. These results suggest that ingested ecdysone might change the sensitivity of epidermis to 20E.

INTRODUCTION

We recently reported that as many as 11 larval ecdyses, i.e. 7 additional molts, which we call "ultranumerary larval ecdyses", can be induced by feeding ecdysone (E) to the silkworm, *Bombyx mori* (Tanaka & Takeda, 1993a) and that the dietary effect of E is qualitatively different from that of 20-hydroxyecdysone (20E) (Tanaka & Takeda, 1993b).

The most apparent difference between the larvae receiving the two ecdysteroids is in their ecdysteroid titer. When the larvae are fed since hatching on diets supplemented with E, the ecdysteroid titer fluctuates, reaching a peak and then declining in each instar. By contrast, in larvae fed 20E, the ecdysteroid titer remains high and the larvae die in the second instar (Tanaka & Takeda, 1993b). In the control or the E-fed larvae, the feeding is interrupted and head capsule slippage occurs (general apolysis) when ecdysteroid titer begins to increase (Kiguchi & Agui, 1981). Most of the larvae receiving 300 ppm 20E from the start of the 4th instar, however die during the 5th instar without undergoing apolysis in spite of high ecdysteroid titer in their hemolymph (Tanaka et al., 1994a).

It seems that ingested E and 20E exert different effects on the apolysis, similarly as was reported for their action on cuticle formation (Oberlander et al., 1973; Quennedey et al., 1983). In this paper we investigated the effects of E and 20E on the induction of apolysis in *Bombyx mori* in detail.

MATERIALS AND METHODS

Insect rearing

The C145 × N140 race of *Bombyx mori* was reared at 25 ± 1°C under a photoperiodic regime of 12 h light and 12 h darkness on the standard artificial diet (Yakult Co. Ltd). At the start of the 4th instar, the larvae were transferred to a diet supplemented with 300 ppm 20E. Consistently with our earlier

observations (Tanaka et al., 1994a), almost all these larvae molted into the 5th instar earlier than the larvae fed the standard diet. These precociously ecdysed larvae were used within 6 h after ecdysis for the present experiments.

Chemicals

Solutions of E or 20E (Sigma) in 5% ethanol were added to the standard diet during its preparation. Concentration of ecdysteroids in the diets are expressed as ppm per dry matter.

Radioimmunoassay of ecdysteroids

Hemolymph samples (10 µl) were collected from the prolegs of larvae and stored at –20°C until extracted with 100% methanol. The extraction and the radioimmunoassay (RIA) of ecdysteroids were performed as described by Takeda et al. (1986). Crossreactivity ratio between E and 20E was 1 : 2.5 (Takeda et al., 1986). Results are expressed as 20E equivalents per ml hemolymph.

High performance liquid chromatography (HPLC)

HPLC analyses of ecdysteroids were done according to Beydon & Lafont (1987). Hemolymph samples (200 µl) were extracted twice with chloroform-distilled water (1 : 1, V/V). Makisterone A (Sigma) was used as internal standard. After centrifugation, ecdysteroids in the aqueous phase were absorbed onto a SEP-PAK C-18 cartridge column (Waters, Milford) and eluted with 60% methanol after previous column washing with distilled water and 25% methanol. HPLC was performed with the EYELA PLC-5D system instrument (Tokyo Rikakikai Co., Japan) equipped with Shimadzu-Shimpack CLC-ODS(M) (15 cm × 6.0 mm) column. Ecdysteroids were eluted with 20% acetonitrile containing 0.1% TFA (rate 1 ml/min) and quantified with RIA.

RESULTS

Precocious 5th instar larvae obtained with 20E were transferred immediately after ecdysis to diets supplemented either with 100–400 ppm E or with 100–500 ppm 20E (Table 1). Apolysis was recognized by head capsule slippage. The first group of experimental larvae molted into the 6th instar earlier than did the control larvae, and mortality was negligible. Larvae of the latter group died during the fifth instar. Almost all larvae receiving 300 ppm 20E continued to feed for a few days and then perished without entering apolysis, whereas 50% of those receiving 500 ppm 20E completed apolysis but could not shed their old cuticle completely and died at ecdysis.

TABLE 1. Effects of ecdysone or 20-hydroxyecdysone on the induction of apolysis. Larvae received in the 4th instar a diet with 300 ppm 20E and since ecdysis into the 5th instar diets with indicated amounts of E or 20E.

Concentration in the diet (ppm)	N	Duration of the 5th instar (days)	Apolysis (%)**	Ecdysis (%)
Control	45	6.50	90.0	90.0
Ecdysone				
100	42	3.77	95.2	95.2
200	42	2.91	84.0	84.0
300	42	2.87	95.2	95.2
400	47	2.70	94.0	94.0
20-hydroxyecdysone				
100	50	4.16*	0	0
200	50	2.91*	0	0
300	72	2.80*	2.8	0
500	60	3.42*	50.0	6.7

* Time to death

** % larvae with head capsule slippage

In larvae transferred to diet with 300 ppm 20E, the total ecdysteroid titer increased to similar levels as in those that were transferred to diet with 200 ppm E (Fig. 1). The amount of 20E in hemolymph was higher than that of the larvae fed with 200 ppm E, but the concentration of E in hemolymph dropped to a very low level (Fig. 2). In larvae receiving 500 ppm 20E, however, the ecdysteroid titer in hemolymph exceeded 1000 ng/ml: the titer of 20E was higher than in the larvae fed 300 ppm 20E, but the E titer was similar to that in larvae which were fed 200 ppm E (Fig. 2).

TABLE 2. Effects of combinations of ecdysone and 20-hydroxyecdysone on the induction of apolysis. Larvae received in the 4th instar a diet with 300 ppm 20E and since ecdysis into the 5th instar diets with indicated amounts of E or 20E.

Concentration in the diet (ppm)		N	Duration of the 5th instar (days)	Apolysis (%)**	Ecdysis (%)
Control		45	6.50	90.0	90.0
E	20E				
50	250	51	3.02*	17.6	0
100	200	69	3.08*	23.2	0
150	150	55	2.46	86.7	12.9
200	100	47	2.57	87.2	60.0

* Time to death

** % larvae with head capsule slippage

To examine the possibility that E was indispensable for the induction of larval apolysis, some larvae were transferred to a diet supplemented with both E and 20E (Table 2). Almost all larvae ingesting 150 ppm E and 150 ppm 20E entered apolysis, but most of them could not shed the old cuticle and died. Sixty percent of the larvae receiving 200 ppm E and 100 ppm 20E accomplished ecdysis to the sixth instar. In larvae fed 200 ppm E and 100 ppm 20E, the hemolymph ecdysteroid titer was similar as in the larvae fed 300 ppm 20E alone (Fig. 3). The titer of 20E in the larvae fed 200 ppm E and 100 ppm 20E, however, was lower than in the larvae fed 300 ppm 20E alone (Fig. 2).

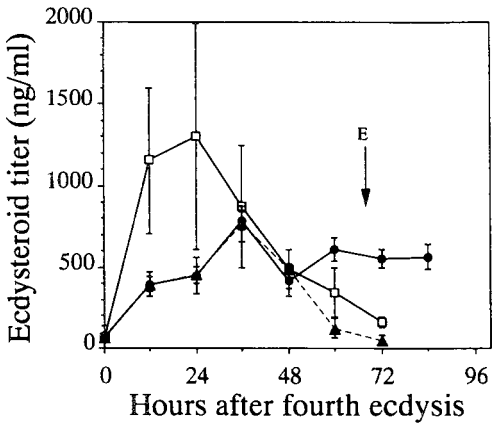

Fig. 1. Changes in hemolymph ecdysteroid levels in larvae precociously molted into the fifth instar and then kept on diets supplemented with E or 20E. ▲ – larvae fed 200 ppm E; ● – larvae fed 300 ppm 20E; □ – larvae fed 500 ppm 20E. Arrow indicates the time in larvae fed 200 ppm E.

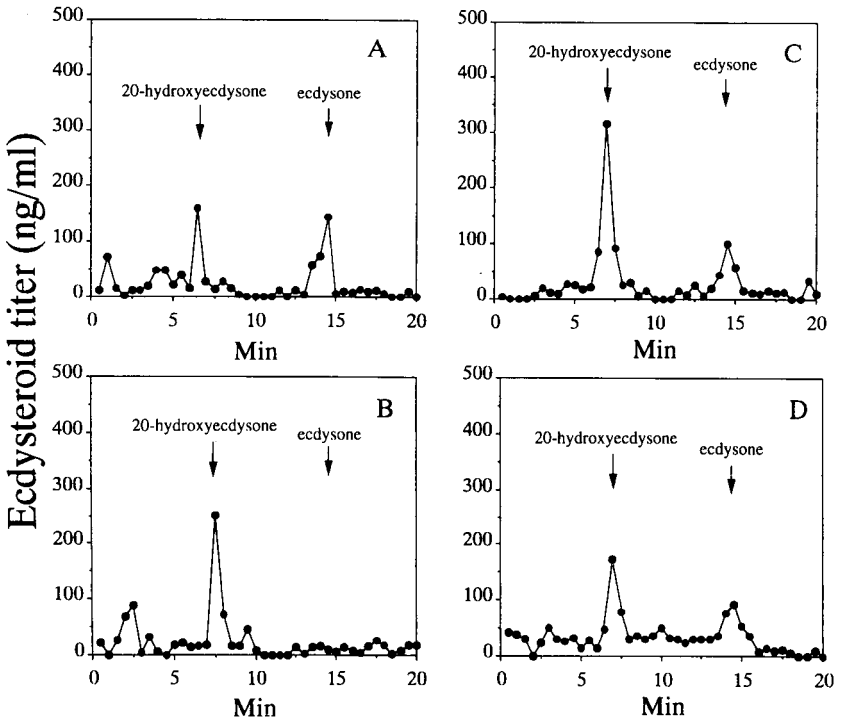

Fig. 2. Hemolymph ecdysteroids at 24 h (D) and 36 h (A, B, C) of the 5th instar in larvae that received in their diet E, 20E or both E and 20E respectively. Ecdysteroids were analyzed by HPLC-RIA. A – larvae fed 200 ppm E; B – larvae fed 300 ppm 20E; C – larvae fed 500 ppm 20E; D – larvae fed 200 ppm E and 100 ppm 20E. Arrows mark elution times of the standard samples.

DISCUSSION

Our results show that E plays a significant role, and different from that of 20E, in the induction of apolysis. Since 20E but not E exerts an inhibitory effect on feeding (Tanaka et al., 1994b), the larvae never ingest large amounts of 20E. However, in larvae fed 300 ppm 20E, a larger amount of 20E is present in the hemolymph than in the larvae fed 200 ppm E, and yet, such a large amount of 20E does not cause apolysis. Identity of the major ecdysteroid fraction in hemolymph with 20E was confirmed using a normal-phase column (data not shown). Such an excessive amount of 20E does not seem to be responsible for the adverse effect since the titer of 20E was lower in larvae fed ≤ 200 ppm 20E ppm than in larvae fed 200 ppm E (data unpublished).

Similar results were obtained in the sweet potato hornworm, *Agrius convoluvli*. When the larvae of *Agrius* feed on diets supplemented with 1,600 ppm 20E, their hemolymph ecdysteroids increase to as high a level as in the larvae fed 800 ppm E, and the titer of 20E in the hemolymph is even much larger, but the larvae receiving 1,600 ppm 20E do not molt earlier than the control larvae (Tanaka & Naya, unpublished).

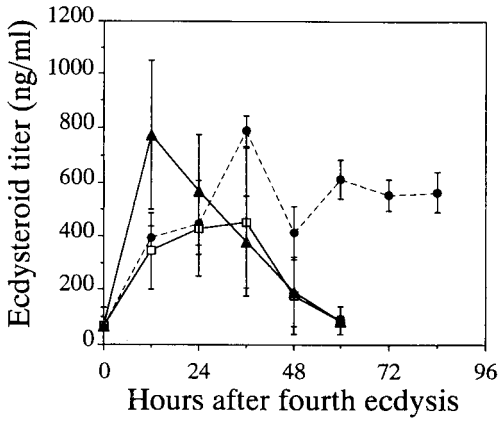

Fig. 3. Changes in hemolymph ecdysteroid level during the fifth instar of larvae transferred to diets supplemented with both E and 20E. ▲ – larvae fed 200 ppm E and 100 ppm 20E; □ – larvae receiving 150 ppm E and 150 ppm 20E; ● – larvae fed 300 ppm 20E alone.

Larval apolysis can be induced in the 5th instar silkworm larvae with 500 ppm 20E. The amount of 20E in the hemolymph of these larvae is higher than in the larvae fed 300 ppm 20E, but E titer also elevated to a comparable level as in the larvae fed 200 ppm E. Furthermore, combinations of E and 20E also resulted in the induction of larval apolysis though the total ecdysteroid level was not so different from that of the non-apolysing larvae fed 300 ppm 20E alone. These results suggest that E or ecdysteroids other than 20E might play important role on the induction of apolysis, and subsequent cuticle formation in *Bombyx*, similarly as reported for other insects (Oberlander et al., 1973; Blais & Lafont, 1980; Caruelle, 1980; Quennedey et al., 1983). The sensitivity of *Bombyx* epidermis to 20E might be altered by E as observed in case of cuticle deposition in *Plodia* (Oberlander et al., 1973). We are currently investigating this possibility by in vitro experiments.

The larvae transferred to 500 ppm 20E enter apolysis but cannot shed the old cuticle completely. The larvae fed both E and 20E also cannot molt into the 6th instar as readily as those receiving E alone. Hence, ingested 20E or its metabolites might have some adverse effect on cuticle formation even in the presence of E.

Bombyx seems to be more sensitive to the ingested ecdysteroids than other insects (Kubo et al., 1983; Tanaka & Naya, in prep.), and provides thus a suitable model for exploring the different effects of E and 20E.

ACKOWLEDGEMENTS. We thank Dr S. Takeda of National Institute of Sericultural and Entomological Sciences, for critical reading of the manuscript. This work was supported in part by a Grant-in-Aid from Ministry of Agriculture, Forestry and Fisheries (BMP-94-5-I-6-3).

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