Role of ecdysteroids in the dynamics of insect haemolymph sugar

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Role of Ecdysteroids in the Dynamics of Insect Haemolymph Sugar

Yasunori Oda[1], Masanori Uejima[1], Masafumi Iwami[1] and Sho Sakurai[1,2]*

[1]*Division of Life Science, Graduate School of Natural Science and Technology and* [2]*Department of Biology, Faculty of Science, Kanazawa University, Kakumamachi, Kanazawa 920-1192, Japan*

ABSTRACT—In *Bombyx* 5th instar larvae, haemolymph trehalose concentrations were maintained in a range of 8-14 mM during the feeding period and then decreased to approximately half the level concomitantly with the occurrence of a gut purge. The decrease occurred as well in larvae that were starved for 29 hr before the gut purge and those that were ligated around the neck. In contrast, ligation between the thorax and abdomen suppressed such a decrease in trehalose concentrations. These results indicated that thoracic factor(s) was involved in the decrease in the haemolymph trehalose concentration. Injections of ecdysteroids into the isolated abdomens of day 5 larvae resulted in a decrease in haemolymph trehalose concentrations. These findings suggest that an increase in the haemolymph ecdysteroid titer at the wondering stage brings about a decrease in the haemolymph trehalose concentrations. Ecdysteroids thus appear to play an important role in the changes in the carbohydrate metabolism at the larval-pupal transformation.

INTRODUCTION

The blood or haemolymph contains free sugars and the levels of the major blood sugar are homeostatically maintained at similar levels. Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a major sugar generally found in insect haemolymph (Mullins, 1985). In Lepidoptera, its levels are maintained homeostatically at similar levels in hours but changes throughout post-embryonic development (Saito, 1963; Wyatt, 1967; Sakamoto and Horie, 1979; Hirano and Yamashita, 1980). In *Bombyx* last larval stadium, trehalose concentrations are maintained at relatively constant levels during the feeding period lasting several days before the onset of the wandering period, after which they sharply decrease from about 10 mM to approximately half the level. After pupation, the concentrations gradually increase and recover almost the same level as found in the feeding period (Oda *et al*., 1997). The changes in the haemolymph trehalose concentrations thus indicate that there are two different control mechanisms, one for homeostatic controls operating within hours and the other for the developmental regulation.

Though the control mechanisms underlying the changes in a haemolymph sugar level within minutes or hours have been extensively studied so far from the point of view of homeostatic regulation during the high-energy demanding behaviour as the flight of adult insects, no information is available for the developmental control of haemolymph sugar levels. The changes in haemolymph trehalose concentrations throughout metamorphosis are probably under the control of hormones that are involved in integrating all developmental events. Ecdysteroid may be one such hormone since various developmental events occurring in the wandering period are always under the control of ecdysteroids as far as is known (Riddiford, 1985). Haemolymph trehalose concentrations decrease concomitantly with the increase in the haemolymph ecdysteroid titer that evokes gut purge (Oda *et al*., 1997). In addition, ecdysone is involved in the regulation of trehalose synthesis (Kobayashi and Kimura, 1966), indicating a relationship between the decrease in trehalose concentration and the increase in ecdysteroid titer. We therefore hypothesized that ecdysteroids are involved in the dynamics of haemolymph sugars. In the present paper, we report evidences for the involvement of ecdysteroid in the decrease in haemolymph trehalose concentration at the larval-pupal metamorphosis.

MATERIALS AND METHODS

Animals

Eggs of *Bombyx mori* were obtained from Aizu Sanshu (Aizuwakamatsu, Fukushima, Japan) and Kanebo Silk Elegance (Kasugai, Aichi, Japan). Larvae were reared on an artificial diet (Nihon Nosan Kogyo) under a 12L:12D photoperiodic regimen at 25°C. Day 0 of the 5th stadium was defined as the 24-hr period starting from the begin-

* Corresponding author: Tel. +81-76-264-5713;
FAX. +81-76-264-5977.
E-mail: ssakurai@kenroku.kanazawa-u.ac.jp

ning of photophase preceded by the 4th ecdysis. Larvae were staged at the time of larval ecdysis and spinneret pigmentation (Kiguchi, 1983; Sakurai et al., 1998). Spinneret pigmentation predominantly occurred during the scotophase of day 5, and gut purge occurred 24 hr after the spinneret pigmentation (Sakurai *et al.*, 1998).

Hormones and chemicals

Trehalose and sucrose were purchased from Wako Pure Chemical Industries (Osaka) and Nakarai (Kyoto), respectively. Bistrimethylsilylacetamide and trimethylchlorosilane used for trimethylsilyl derivertization were obtained from Wako Pure Chemical Industries. Ecdysone and 20-hydroxyecdysone (20E) (Sigma) were dissolved in ethanol, and the concentration was spectrophotometrically determined at 243 nm. After the solvent was evaporated, ecdysteroids were dissolved in distilled water for injections. All other chemicals were of reagent grade.

Ligations and injections

Larvae were anesthetized with diethyl ether prior to ligation or injection. Larvae were tightly ligated with cotton thread followed by cutting the portion anterior to the ligature. Ecdysteroid solution was injected through a proleg after ligation, and the injured proleg was ligated with cotton thread. Distilled water was injected as a control. Haemolymph was collected from the same larva immediately before ligation and at the indicated times.

GLC analysis of trehalose

Preparation of haemolymph samples and quantitative analysis of haemolymph trehalose by gas-liquid chromatography (GLC) were performed as described previously (Oda *et al.*, 1997).

RESULTS

Effects of ligation on trehalose concentrations

Larvae were starved or ligated between the head and thorax or the thorax and abdomen at the beginning of photophase of day 2–6. Haemolymph samples were collected immediately before and 12 and 24 hr after treatments (Fig. 1), except for day 2 when haemolymph samples were collected immediately before and 12 and 23 hr after treatments. Haemolymph trehalose concentrations immediately before the treatments were ranged from 8–14 mM. When day 2 larvae were starved, the trehalose concentrations decreased from 13.4 to 10 mM in 12 hr of starvation whereas the trehalose concentrations in the isolated abdomens decreased to 19.1% of initial levels (2.4 mM) 12 hr after the isolation. On day 3, the trehalose concentrations greatly decreased in the isolated abdomens as well as the neck-ligated larvae, similar to the decrease in day 2 abdomens, but the decrease in the starved larvae was not pronounced. In abdomens isolated on day 4, the trehalose concentrations slowly decreased after isolation. A similar decrease was recorded in the neck-ligated larvae, but such a decrease was not recorded on day 5. On day 4 or 5, the concentrations in the starved larvae remained at the initial level or transiently increased at 12 hr. On day 6, the trehalose concentrations decreased in all the preparations, i. e. starved larvae, neck-ligated larvae, and isolated abdomens.

If the decreases in trehalose concentrations on day 6 in all the preparations were brought about by the haemolymph ecdysteroids that had been released for inducing a gut purge, larvae before the time of the increase in haemolymph ecdysteroids must be used for examining the involvement of ecdysteroids in changes in haemolymph trehalose concentrations. Accordingly, we ligated larvae between the head and thorax or the thorax and abdomen 3 hr before the beginning of the photophase of day 6. As shown in Figure 2, trehalose concentrations in the isolated abdomens were maintained at initial levels for 29 hr after isolation. In contrast, the concentrations in both the starved larvae and neck-ligated larvae decreased to approximately 40% of initial levels during the same period.

Effects of ecdysteroid on trehalose concentrations

Since the trehalose concentrations did not decrease in the abdomens that were isolated on days 4 and 5 (see Fig. 1), 20E effects on trehalose concentration were examined using abdomens isolated on these days. Abdomens were isolated

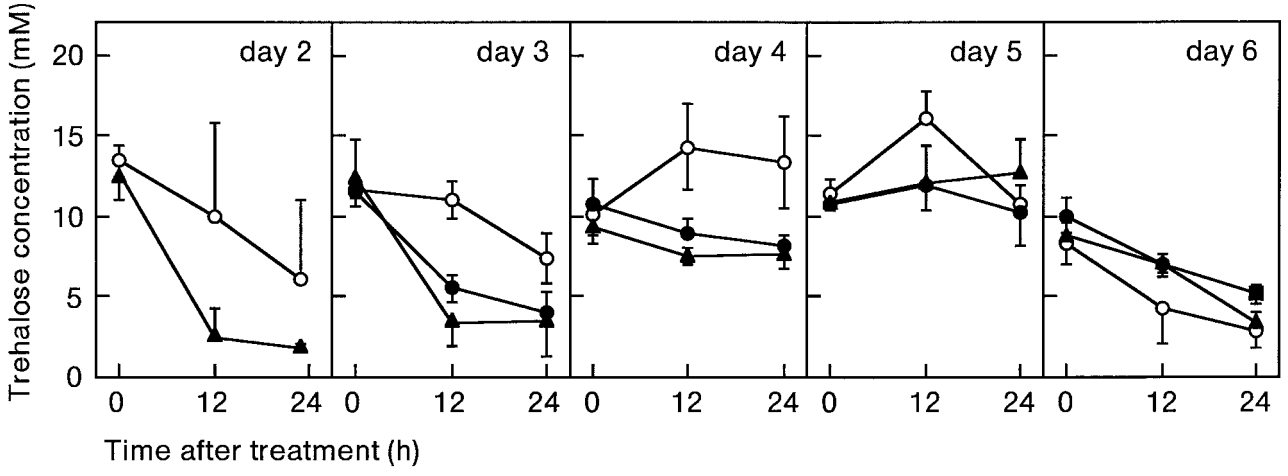

Fig. 1. Developmental effects of starvation, neck-ligation, and isolation of abdomens on haemolymph trehalose concentrations. Larvae were starved (open circles), neck-ligated (closed circles), or ligated between the thorax and abdomen (closed triangles) at the beginning of photophase of each of days 2, 3, 4, 5, and 6 in the 5th stadium. Haemolymph was collected three times from the same larva. Each datum point indicates a mean ± SD of 3–5 individual determinations.

at the beginning of photophase of each day and immediately injected with 20E (0.2 μg/g body weight) or distilled water as a control (Fig. 3). On day 4, trehalose concentrations in the control abdomens decreased to approximately half of initial levels in 12 hr and then slightly increased at 23 hr. Trehalose concentrations in the 20E-injected larvae at 23 hr was significantly lower than that in the control larvae (P<0.05, Student's t-test). On day 5, trehalose concentrations in the control abdomens remained at initial levels for 12 hr after the isolation while 20E effectively decreased the concentration to approximately a half that of initial levels in 12 hr.

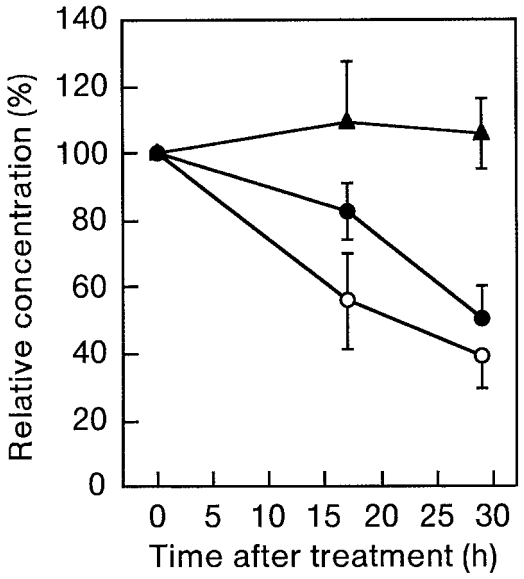

Fig. 2. Effects of neck-ligation and ligation between the thorax and abdomen on haemolymph trehalose concentrations. Larvae were starved (open circles), neck-ligated (closed circles), or ligated between the thorax and abdomen (closed triangles) 3 hr before the beginning of photophase of day 6. Haemolymph was collected three times from the same larva i. e., immediately before (0 h) and 17 and 29 hr after the treatments. The values for the trehalose concentrations are shown as percentages of the concentration immediately before the injection. Each datum point indicates a mean ± SD of 5 individual determinations.

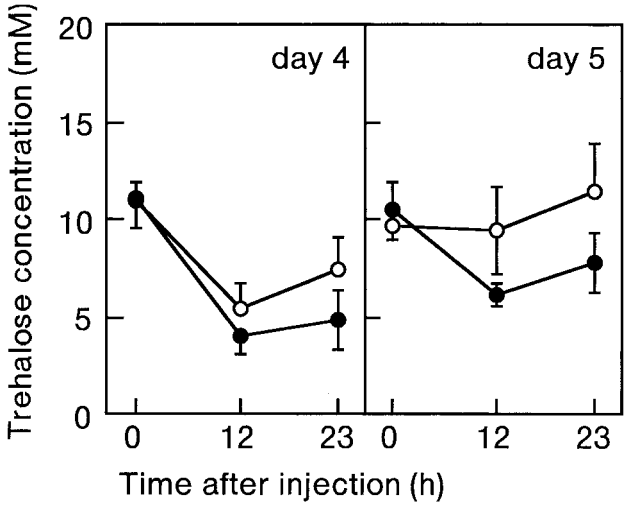

Fig. 3. Effects of 20E on haemolymph trehalose concentrations. Larvae were ligated between the first and second abdominal segments followed by injection with 10 μl water (open circles) or 20E (0.2μg/g body weight) (closed circles) at the beginning of photophase of days 4 and 5 of the 5th stadium. Haemolymph was collected three times from the same larva, i. e. immediately before and 12 and 23 hr after the treatments. Each datum point indicates a mean ± SD of 5 individual determinations.

Dose-response for ecdysteroid

Various doses of 20E were injected into abdomens isolated at the beginning of each photophase of days 4 and 5. Haemolymph was collected immediately before and 24 hr after the injections. The dose-response for ecdysone was also examined for day 4 larvae. In day 4 abdomens, the haemolymph trehalose concentrations decreased in a dose-dependent manner for both ecdysone and 20E (Fig. 4 a). In order to compare the effectiveness of ecdysone and 20E, we estimated the critical dose based on the dose-response curve. The critical doses for 20E and ecdysone were 0.22 and > 0.5 μg, respectively. Since the effects of ecdysone were not satu-

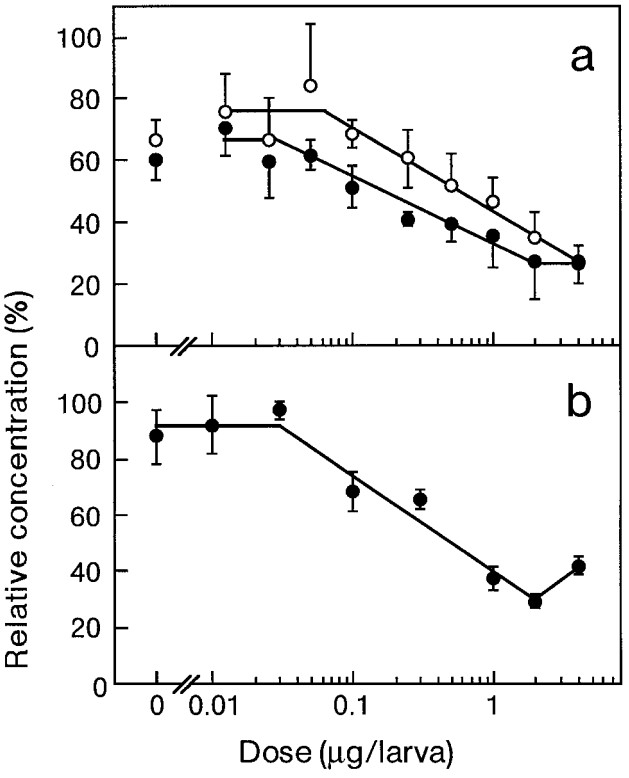

Fig. 4. Dose-response of haemolymph trehalose concentrations for the injected ecdysteroids in abdomens isolated on day 4 (a) or 5 (b). Larvae were ligated between the first and second abdominal segments and then injected with various doses of 20E (closed circles) or ecdysone (open circles in a). Haemolymph was collected twice from the same larva, i. e. immediately before and 24 hr after the injections. The values for the trehalose concentrations are shown as percentages of the concentration immediately before the injections. Each datum point indicates a mean ± SD of 5 individual determinations.

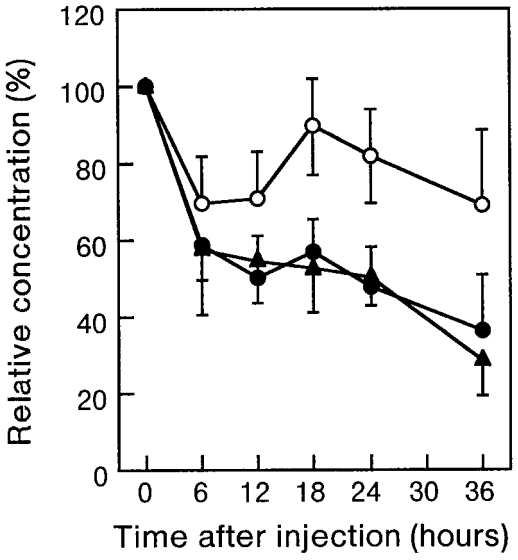

Fig. 5. Time-course of the decrease in haemolymph trehalose concentrations after injection of ecdysteroids. Isolated abdomens of day 4 larvae were injected with 10 μl of distilled water as a control (open circles), 1 μg ecdysone (closed circles), or 1 μg 20E (closed triangles). Larvae were divided into 3 groups according to the time schedule of haemolymph collection. Haemolymph was collected at 0, 6, and 18 hr from the first group larvae, at 0, 12 and 24 hr from the second group larvae, and at 0 and 36 hr from the third group larvae. The data of each group are expressed as percentages of the concentration before the injections in the same group and then combined for depicting the curves. Each datum point indicates a mean ± SD of 5 individual determinations.

rated at 4 μg, the critical dose value for ecdysone indicates the minimum one, and is probably larger than the calculated value. The dose-response curve depicted on day 5 (Fig. 4 b) was similar to that on day 4, and the critical dose was 0.22 μg.

Time-response curves for ecdysteroids were obtained by injecting 1 μg ecdysone or 1 μg 20E into the isolated abdomens of day 4 larvae. For the first 6 hr after the injection of ecdysone or 20E, the haemolymph trehalose concentrations decreased sharply to approximately 60% of initial levels but were not significantly lower than those in the control, water injected abdomen. From 6 hr, the trehalose concentrations gradually decreased to approximately 30% of initial levels at 36 hr, whereas the concentrations in control larvae remained at similar levels until 36 hr with fluctuations between 70 and 90%.

DISCUSSION

In the present study, we showed that the effects of ligation on haemolymph trehalose concentrations changed day by day during the feeding period of the 5th stadium. On days 2 and 3, the haemolymph trehalose concentrations decreased remarkably in the isolated abdomens as well as neck-ligated larvae, while the concentrations in larvae starved for 12 hr showed only a slight decrease, indicating that head factor(s) is required for maintaining the trehalose concentration on those days. The remarkable decrease in the haemolymph trehalose concentrations after ligation was not reproduced in day 4 and 5 larvae. It is possible that the requirement of a head factor(s) for maintaining haemolymph trehalose concentration is limited to the early feeding period in the 5th stadium.

The different effects of the ligation along with larval development may reflect different physiological states with relation to syntheses of trehalose and glycogen in fat bodies. Fat body trehalose synthase activity is low on day 0 and remarkably increases from day 0 to day 4 (Hirano and Yamashita, 1983). On the other hand, total glycogen synthase activity is high on day 2 and 3 but sharply decreases to a low level from day 3 to day 4 (Hattori and Iwami, unpublished data). Thus a head factor(s) possibly enhances the trehalose production to retain constant haemolymph trehalose concentrations at the time when the trehalose synthase activity is low and the glycogen synthase activity is high. One possible head factor is thought to be adipokinetic hormone (AKH) because *Bombyx* AKH possesses hypertrehalosemic activity (Oda and Uejima, unpublished data). Since the trehalose concentrations scarcely decreased either in the isolated abdomens or neck-ligated larvae on days 4 and 5, the involvement of head factor(s) was uncertain on these days.

On day 6, the trehalose concentrations decreased equally in three preparations, i. e. starved larvae, neck-ligated larvae and isolated abdomens, which were prepared at the beginning of photophase on day 6. In the present study, we used Gate I larvae that showed gut purge in the scotophase of day 6. Accordingly, the time when day 6 larvae were treated was the time when haemolymph ecdysteroid titer had begun to increase for triggering gut purge (Sakurai *et al.*, 1998). If the increased haemolymph ecdysteroids decreased haemolymph trehalose concentrations on day 6, it can be concluded that there were no differences in the trehalose concentrations after the above three treatments. We therefore used the larvae 3 hr before the beginning of photophase of day 6, which was immediately before the beginning of an increase in the ecdysteroid titer. Ligation between the thorax and abdomen significantly interrupted the decrease in haemolymph trehalose concentrations that occurred in the control larvae. Neck-ligation, however, did not interrupt such a decrease. These results indicate the involvement of the thorax but not the head in the decrease in haemolymph trehalose concentrations occurring at the end of the feeding period.

We showed that ecdysteroids may be the thoracic principle that decreases the haemolymph trehalose concentrations at the onset of the wandering stage. Gut purge is one of the ecdysone-dependent events (Truman and Riddiford, 1974) in the course of pupal metamorphosis, and haemolymph ecdysteroid concentrations increase approximately 12 hr before the gut purge (Sakurai *et al.*, 1998). A decrease in the haemolymph trehalose concentrations begins approximately 12 hr prior to gut purge (Oda *et al.*, 1997), strongly indicating that ecdysteroid is the thoracic factor for the decrease in trehalose concentrations. 20E exerted a hypotrehalosemic effect in day 4- and 5-isolated abdomens. The critical dose of 0.22 μg/larva for 20E was approximately 10 times higher than

the haemolymph ecdysteroid concentration at the time of gut purge (Sakurai *et al.*, 1998), but is not high if compared with the doses required for evoking other ecdysteroid-dependent developmental events. Haemolymph ecdysteroid concentration can be tentatively elevated by a single injection of 20E but promptly decline to initial level in less than 3 hr (Nagata *et al.*, 1987). The dose corresponding to a haemolymph ecdysteroid titer is not therefore sufficient for inducing such events. Artificial induction of ecdysteroid-dependent events need a single injection of an amount that is much larger than the physiological concentration or repeated injections (Zdarek and Fraenkel, 1970) or continuous infusion (Nijhout, 1976) of a physiological dose. In day 4-isolated abdomens, ecdysone was 2-fold less effective than 20E (Fig. 4). 20E is similar to or more potent in evoking biological events than ecdysone (Cherbas *et al.*, 1980), and a difference in the effective doses of ecdysone and 20E is usually observed for most biological assays of ecdysteroids.

The decreases in the haemolymph trehalose concentrations at the time of wandering (Oda *et al.*, 1997) may be due to a reduction in trehalose synthase activity in the fat body (Hirano and Yamashita, 1980; 1983). Thus the hypotrehalosemic activity of ecdysteroids as revealed in the present study may be related to the reduction in trehalose production. In contrast, ecdysone enhances the trehalose synthesis in the brainless *Bombyx* pupae (Kobayashi and Kimura, 1966). Accordingly, the effects of ecdysteroids on the trehalose dynamics change along with insect growth and metamorphosis. The present study is the first to show that ecdysteroids play an important role in regulating energy metabolism as well as morphogenesis throughout insect development.

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