INTRODUCTION

Biliverdin is a kind of bile pigments and a common component in many insects. The function of biliverdin is not well understood, but the green coloration resulting from a combination of blue biliverdin and yellow carotinoid in the hemolymph or epidermis of caterpillars sometimes camouflages the larvae on green plants. Biliverdin usually binds to the specific proteins in the hemolymph and the biliverdin-binding proteins with different molecular masses have been isolated from many insect species (Cherbas, 1973; Chino et al., 1983; Haunerland and Bowers, 1986; Jones et al., 1988; Chinzei et al., 1990; Yoshiga and Tojo, 1995; Saito and Shimoda, 1997; Saito, 1998a, b; Saito et al., 1998).

In Spodoptera litura, we have isolated four biliverdin-binding proteins (BPs, in the order of highest isoelectric point, BP-1, BP-2, BP-3 and BP-4), which are composed of 165 kDa subunits, contain carbohydrates and lipids, and mainly exist in the larval hemolymph (Yoshiga and Tojo, 1995). The function of BPs is not clear, but its lipid components and the sequestration by fat body shortly before pupation associate one of their functions with both amino acid and lipid reservoir. They can be classified immunologically into two groups, and the hemolymph titers of these groups change differently: BP-A (BP-1, BP-2 and BP-3) exists mainly in the sixth instar, while BP-B (BP-4) is predominant in the larvae before the sixth instar (Yoshiga et al., 1998). The difference of the hemolymph titers indicates that BP-A and BP-B are synthesized under different hormonal milieu, which seems to depend largely on juvenile hormone (JH). To elucidate the possible different responses of BP titers to JH, we applied a JH analog, methoprene, to the fifth or the sixth instar of S. litura, and examined the effects on the BP titers in the hemolymph.

Effects of a juvenile hormone analog, methoprene, on the hemolymph titers of biliverdin-binding proteins in the common cutworm, Spodoptera litura (Lepidoptera: Noctuidae)

Toyoshi Yoshiga* and Sumio Tojo

Laboratory of Applied Entomology, Department of Applied Biological Sciences, Faculty of Agriculture, Saga University, Saga 840–8502, Japan

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Abstract

Effects of methoprene (a juvenile hormone analog) on the larval development and hemolymph titers of biliverdin-binding proteins (BPs) in Spodoptera litura were studied. BP-A first appeared in the hemolymph of the fifth (penultimate) instar larvae, decreased during the molting to the sixth instar and prominently increased during the sixth (last) instar. On the other hand, the titer of BP-B, a predominant component in preceding stadia, greatly increased during the fifth instar, drastically decreased before the ecdisis to sixth instar, and slightly increased during the sixth (last) instar. Methoprene (0.1–5 μg/insect) topically applied on day 0 of the fifth instar caused little effect on the duration of the fifth instar and on the titers of BPs, while the application to day 0 sixth instars extended the last instar dose-dependently and changed the BP titers as follows: BP-A showed a similar increase as in untreated control, but continued to increase further during the prolonged feeding period; BP-B titer showed a increase within one day of the application. Double applications of methoprene at one day interval induced a stepwise increase of the BP-B titer at each application, but not of BP-A. These results suggest that BP-B synthesis is triggered by juvenile hormone, which also regulates the synthesis of BP-A indirectly by blocking larval-pupal transformation.

Key words: Spodoptera litura, methoprene, juvenile hormone, biliverdin, storage protein

* To whom correspondence should be addressed at: E-mail: tyoshiga@cc.saga-u.ac.jp
MATERIALS AND METHODS

**Insect rearing.** Larvae of *S. litura* were reared at 25°C under a 16 h light and 8 h dark photoregime (lights-on at 8:00 and lights-off at 24:00) on an artificial diet (Okada, 1977). To synchronize the larval development, larvae at head capsule slippage were collected about 8 h before lights-off. The collected insects were placed in plastic cups (8 cm diameter and 4.5 cm height) and maintained without food. Shortly after lights-on on the next day, an artificial diet was given to newly ecdysed larvae. Rearing densities were 200, 50, 20, 10 and 5 larvae per a cup at the third, fourth, fifth, sixth and seventh (supernumerary) stadia, respectively.

**JH analog application.** A juvenile hormone analog, methoprene (91% purity, Otsuka Chemical Co.), was used for the experiments. Five microliters of the solution containing various concentrations of methoprene diluted with 99% ethyl alcohol was topically applied onto the dorsal abdomen of each larva at 8:00 of day 0 fifth, day 1 fifth, day 0 sixth, or at 24:00 of day 1 sixth larval stadium. In some experiments, two successive applications were carried out at one day interval.

**Hemolymph collection.** Hemolymph was bled into ice-chilled tubes (0.5 ml) containing a few crystals of phenylthiourea (PTU) by cutting the legs of the larva. After removal of hemocytes and debris by centrifugation at 5,000×g for 10 min at 4°C, the supernatant was diluted fourfold with phosphate buffered saline (PBS: 200 mM potassium phosphate, 150 mM NaCl, pH 7.0) containing 5 mM Na-EDTA, 0.5 mM phenylmethyl sulfonyl fluoride (PMSF), and 2 mM dithiothreitol (DTT), and then stored at −70°C until use. The hemolymph was collected at 16:00 of each day.

**Tissue preparation.** For tissue sampling, larvae were dissected out in ice-cold PBS, then fat body, heart and carcass were collected, rinsed well with cold PBS. The tissues were homogenized with 10× vol of PBS containing 5 mM Na-EDTA, 0.5 mM PMSF, 2 mM DTT and a few crystals of PTU, and centrifuged at 5,000×g for 10 min at 4°C. The supernatants were subjected to rocket immunoelectrophoresis.

**Rocket immunoelectrophoresis.** Antisera against BPs were prepared previously (Yoshiga and Tojo, 1995). Agarose gels (0.8%) containing 60 mM veronal buffer (ionic strength=0.05, pH 8.6), 1% antiserum against BP-2 (anti-BP-A) and 0.5% antiserum against BP-4 (anti-BP-B) were used for rocket immunoelectrophoresis. Amount of BPs were calculated by comparing of the heights of rockets with those of purified BPs. A student’s t-test was used for statistical analysis.

**Protein determination.** Protein contents were determined by a protein-dye assay with Coomassie Brilliant Blue G 250 (Bearden Jr, 1978).

RESULTS

**Fluctuation patterns of biliverdin-binding proteins in the hemolymph**

Figure 1 shows the changes of BPs titers in the hemolymph of the fifth and sixth (last) instars. BP-A was hardly detected in the hemolymph of the fifth instar, but the level increased considerably during the sixth larval stadium, and attained a maximum after the time of gut purge. In contrast, the BP-B titer was high in the late fourth instar, but it decreased during the molt to the fifth instar. BP-B increased again during the fifth instar and attained a peak in the late fifth instar. During the ec dysis to the sixth instar, BP-B abruptly declined, showed gradual increase thereafter and reached a peak after...
Effects of methoprene treatment on BP titers during the fifth and sixth stadia

Methoprene (0.1–5 μg/insect) topically applied on day 0 fifth instars did not affect the duration of the fifth stadium (data not shown). The BP-A titers of the methoprene-treated larvae did not differ from those of control, while the concentration of BP-B slightly but significantly increased one day after the methoprene-treatment (5 μg) \( (p<0.05) \) and two days after the treatments (1, 2 or 5 μg) \( (p<0.05) \) (Fig. 2). Application of methoprene on day 0 fifth instar did not affect the BP titers in the hemolymph of the sixth instar (data not shown).

When methoprene was applied (0.5–5 μg/insect) on day 0 sixth instars, the feeding period was extended dose-dependently, resulting in the elongation of the sixth larval stadium from five days for untreated larvae to six days (0.1–0.2 μg), seven days (0.5 μg), eight days (1–2 μg) or nine days (5 μg). These larvae eclosed to bigger pupae. The concentration of BP-A in the hemolymph of methoprene-treated larvae increased in a manner similar to control larvae until day 3 (Figs. 3A and 4A). It increased further up to the time of gut purge, while BP-A in the control hemolymph showed a decline one day later \( (p<0.05) \) (Fig. 4A). On the other hand, methoprene induced a higher concentration of BP-B in the hemolymph (Fig. 4B) somewhat dose-dependently, one day after the treatment \( (p<0.01, \text{control vs } 0.5–5 \mu g \text{ methoprene}) \) and three days after the treatment \( (p<0.05, \text{control vs } 5 \mu g \text{ methoprene}) \) (Fig. 3B). When 1 μg of methoprene was applied on day 0, the BP-B titer increased by day 1, the level was kept until pupation and decreased before pupation (Fig. 4B).

Single or double applications of 1 μg of methoprene on day 0 and day 1 did not affect the level of BP-A until day 3, but the titers increased continuously during the prolonged feeding period thereafter (Fig. 5A). Application of 1 μg of methoprene on day 0 induced an increase in the concentration of BP-B one day later \( (p<0.01) \), and an additional application on day 1 increased the concentration further \( (p<0.01) \); these higher levels were maintained thereafter (Fig. 5B).

Application of 5 μg methoprene to sixth instar larvae at 24:00 on day 1 induced an extra larval-larval molt but these larvae failed to pupate. Titers of both BPs did not appear to be affected during the sixth stadium by the treatment except for an earlier decline of BP-A on day 3 \( (p<0.05) \) and an abrupt increase of BP-B one day after the application \( (p<0.05) \). In the supernumerary eclosed larvae, the hemolymph levels of both BP-A and BP-B were as high as in the sixth instars for at least four days. The level of BP-B however declined to one
gut purge (day 3).

Effects of methoprene treatment on BP titers during the fifth and sixth stadia

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half two days later (Fig. 6).

**Effects of methoprene treatment on BPs in tissues**

BPs were detected in the fat body and pericardial cells of control and methoprene-treated larvae after the time of gut purge at the late sixth stadium but were not during the larval-larval moltings or the feeding periods (data not shown).

**DISCUSSION**

In the present study, we demonstrated that the hemolymph titers of two biliverdin-binding proteins (BP-A and BP-B) in *S. litura* were differently influenced by the treatment of a JH analog, methoprene. The fluctuation pattern of BP-A was not changed by the methoprene treatment at any stages of the fifth and sixth instars while the elongation of the feeding period induced by the treatment al-
followed the continuous increase of the BP-A concentration to a higher level than in the untreated control (Fig. 4A). This type of change is quite similar to that of a storage protein, arylphorin (SL-3) in *S. litura* (Tojo et al., 1985a; Zheng et al., 2000). Arylphorin is the major larval hemolymph protein commonly found in insects and the synthesis is not affected by JH (analog) in any insect species studied (for reviews, see Levenbook, 1985; Kanost et al., 1990). These facts suggest that the synthesis of BP-A is not directly regulated by JH.

On the other hand, the methoprene treatment during the sixth stadium increased the BP-B concentration dose-dependently to as high a level as in...
the fifth instar. Previous works (Tojo et al., 1985a, b; Morita et al., 1988) have given the evidence that the JH titer declines before head capsule slippage of the fifth instars in *S. litura* and attains a lower level by day 1 of the sixth instar to allow the process of larval-pupal commitment by ecdysteroids. In fact, juvenile hormone II, which was recently identified in *S. litura* hemolymph, declined under detectable level by day 1 (Zheng et al., 2000). The JH titer of fifth instar may be already high enough to bring any effects on the syntheses of BPs, so that their syntheses are scarcely influenced by the methoprene treatment. However, after the ecdysis to the sixth instar, the declined JH titer seems not to activate the BP-B synthesis, resulting the low BP-B titer in control larvae, while topical application of methoprene to the last instars under low endogenous JH titer appears to induce the BP-B synthesis. These facts suggest that the BP-B synthesis is controlled by JH during the larval stadia.

Although the methoprene treatment had some influences on the BP titers in the hemolymph of the sixth instars, the hemolymph titers of BPs in the methoprene-induced supernumerary larvae remained constant. Most of these larvae had patches of pupal cuticle around the mouth parts, which made them unable to feed, or pupate. When the larvae were starved from the beginning of the sixth stadium, BPs in the hemolymph did not increase and the larvae died by day 4 (data not shown). Probably, the syntheses of BPs in the supernumerary larvae are stopped by a shortage of a nutrient supply owing to the malformed mouth, indicating that nutrient supply is a must for the synthesis of BPs.

Since BPs in the hemolymph were not decreased by the methoprene treatment, there seems to be no apparent effect of methoprene on the decrease of BPs from the hemolymph. In the previous paper, we reported that BP concentrations in the fat body increased during the late sixth stadium corresponding to the decrease of BPs in the hemolymph (Yoshiga et al., 1998). BPs taken up and stored by the prepupal fat body are gradually decreased in the fat body during the pupal stadium, which seems to be used for the pupal-adult development (Yoshiga et al., 1998). In the present study, BPs were detected in the tissues of methoprene-treated larvae after gut purge but not during the larval-larval molting and the feeding periods, although BPs in the hemolymph decreased at the end of each larval stadium. The decreased BPs from the hemolymph during the larval-larval molting could be the result of rapid sequestration and degradation of the proteins by the larval tissues for the larval development. The function of BPs are still not clear, but these different fluctuation patterns and regulation of BPs support the idea of different roles of BPs: BP-A could be one of the amino acid and lipid sources for the pupal-adult development and BP-B may be mainly used for the larval development.

In addition to BPs, we have purified and characterized three storage proteins (SL-1, SL-2 and SL-3) from the larval hemolymph of *S. litura* (Tojo et al., 1985a; Tojo and Yoshiga, 1994; Zheng et al., 2000). Storage proteins are the major hemolymph proteins during larval stadia, mainly synthesized by the fat body and used for the pupal-adult development (for reviews, see Levenbook, 1985; Kanost et al., 1990). The syntheses of the three storage proteins in *S. litura* are also differently influenced by JH (Tojo et al., 1985a; Zheng et al., 2000). In the larval hemolymph of *S. litura*, there seems to be three different protein groups with regard to JH-sensitivity: JH-suppressive proteins, SL-1 and SL-2; JH-inducing protein, BP-B; JH-non-sensitive proteins, SL-3 (arylphorin) and BP-A. These proteins can be good targets to elucidate the mechanism of endocrinological regulation under different hormonal milieu.

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