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Diapause hormone terminates larval diapause in the bamboo borer, *Omphisa fuscidentalis* (Hampson)

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ABSTRACT

The larvae of the bamboo borer (*Omphisa fuscidentalis* Hampson) maintain a diapause state for approximately nine months. However, changes in the ecdysteroid titer lead to the termination of larval diapause when juvenile hormone analogue (JHA) is applied. The hormonal mechanisms that terminate larval diapause are still unknown. Recently, it was found that the *O. fuscidentalis* diapause hormone and pheromone biosynthesis activating neuropeptide genes (*Ompfu-DH-PBAN*) were expressed during the development of both larvae and pupae and during the late larval diapause. This result suggested that diapause hormone (DH) might be involved in the termination of larval diapause. Thus, our study aims to determine the effect of DH on diapause termination in the bamboo borer. The response of diapausing larvae to DH was assayed using the synthesized *O. fuscidentalis* diapause hormone (Ompfu-DH). After injection with different concentrations of DH (25, 50 and 100 ng/larvae), nearly half of the individual larvae (approximately 45%) became completed or incompleted pupae within 30 days. The mean days of pupation in larvae injected with 25, 50 and 100 ng DH were 26.81 ± 17.86, 26.30 ± 16.45 and 25.30 ± 16.12, respectively. The hemolymph ecdysteroid titer in Ompfu-DH injected-larvae was significantly higher 10–15 days after Ompfu-DH injection and reached a maximum before the formation of the pupal cuticle. These results indicate that Ompfu-DH terminates larval diapause in *O. fuscidentalis* by increasing the ecdysone in hemolymph, reflecting a new role of DH in the regulation of larval diapause in this species.

Introduction

Diapause can occur in any stage of the insect life cycle (egg, larvae, pupa or adult), depending on the insect species. This suspended development event is vital for insects in response to unfavorable environmental conditions. Larval diapause is best known in the Lepidoptera (Fields and McNeil, 1988; Lu et al., 2013; Chippendale and Yin, 1979) and during the last larval instar. Studies on larval diapause have focused primarily how environmental conditions regulate its occurrence (Rock et al., 1983; Li et al., 2003; Chen et al., 2011). In addition, larval diapause occurs as a consequence of neuroendocrine system interactions (Denlinger, 2002). Various hormones are responsible for the maintenance and termination of larval diapause. Juvenile hormone (JH) is an important hormone that is produced by the corpora allata (CA). JH regulates insect development; the progression of larval diapause is determined by the amount of JH in the hemolymph. An increase in the level of JH in the hemolymph was found to initiate and maintain larval diapause in the rice stem borer, *Chilo suppressalis* (Yagi and Fukaya, 1974), the Mediterranean corn borer, *Sesamia nonagrioides* (Eizaguirre et al., 1998), and the beet webworm, *Loxostege sticticalis* (Lepidoptera: Pyralidae). Moreover, exogenous juvenile hormone (JH) applied to mature larvae of the southwestern corn borer, *Diatraea grandiosella*, induced diapause and prolonged the larval diapause stage (Yin and Chippendale, 1973). Conversely, a decline in JH resulted in the termination of larval diapause (Jiang et al., 2011).

In addition to JH, ecdysteroid (the principal molting hormone) is another important hormone that controls larval diapause. Larval diapause is maintained by the low level of ecdysteroid in the hemolymph. An elevation in the ecdysteroid titer resulted in the termination of diapausing larvae in *Ostrinia nubilalis* (Gelman and Woods, 1983). Thus, termination of larval diapause can occur by an increase in the ecdysteroid titer. Ecdysteroid is secreted from the prothoracic glands (PGs) via stimulation of the prothoracicotropic hormone (PTTH) by the brain (McBrayer et al., 2007; Endo et al., 1997).

The bamboo borer, *Omphisa fuscidentalis* Hampson, is a moth in the order Lepidoptera. The fifth (final) instar larvae enter diapause in September and pupate in June. Termination of larval diapause in the bamboo borer is influenced by changes in the hemolymph ecdysteroid titer. During the extended larval diapause, the ecdysteroid in the hemolymph is low but increases and remains high during pupation

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(Singtripop et al., 1999). Larval diapause maintenance and termination have been studied by observing the morphological changes induced by exogenous ecdysteroids and their effect on the percentages of pupation and day of pupation. When diapausing larvae were injected with exogenous 20-hydroxyecdysone (20E), they produced tanned pupal cuticles. Moreover, the responsiveness of diapausing larvae to different doses of 20E varied each month. When early diapausing larvae (October–December) were injected with 20E, the day of pupation was the same regardless of dose (Singtripop et al., 2002a). Although JH titer during the development of *O. fuscidentalis* has not been determined, a previous study found that the expression level of juvenile hormone binding protein (*JHBP*) encoding gene in the fat body was high in the diapause period and low from the late-diapause until pupation (Ritdachyeng et al., 2012). These suggest that the larval diapause of *O. fuscidentalis* might be maintained by a low level of ecdysteroid and a high concentration of JH in the hemolymph. However, when juvenile hormone analogue (JHA) was applied to the diapausing larvae, diapause was terminated through an indirect activation of PGs (Singtripop et al., 2000) and increased the hemolymph ecdysteroid level (Singtripop et al., 2002b). Moreover, several genes affect the ecdysteroid level in *O. fuscidentalis*, such as ecdysone receptor gene (*OfEcR-A* and *OfEcR*-B1) and ecdysone-inducible genes (*OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3*) (Suang et al., 2017) and consequently terminated larval diapause. However, the factors that affect the hemolymph ecdysteroid titer in the bamboo borer are unknown.

Diapause hormone (DH) is a neuropeptide hormone that is secreted from the subesophageal ganglion (SG) and is best known for initiating embryonic diapause in the silkworm, *Bombyx mori* (Yamashita, 1996). Although the role of DH in larval diapause is unclear, the effects of DH have been well described in the pupal diapause of the tobacco budworm, *Heliothis virescens* (Xu and Denlinger, 2003) and *Helicoverpa zea* (Zhang and Denlinger, 2012). Larval and pupal diapause are characterized by the cessation of PGs, which fail to synthesize the ecdysteroids that promote development (Denlinger, 1985). Moreover, DH injection increased the hemolymph ecdysteroid titer in pupal diapause (Zhang et al., 2004). Although the mechanism by which ecdysteroid hormone controls larval diapause is similar to pupal diapause, the specific physiological functions of DH that regulate larval diapause in the bamboo borer are unknown.

Recently, Suang et al. (2015) found evidence of *O. fuscidentalis* diapause hormone and pheromone biosynthesis activating neuropeptide (*Ompfu-DH-PBAN*) expression in the SG during larval and pupal development. The expression level of *Ompfu-DH-PBAN* was high during the larval diapause stage and reached its maximum level in late diapause. After pupation, the expression level sharply decreased. Accordingly, the increased expression of *Ompfu-DH-PBAN* before the pupal stage may be associated with an increase of the ecdysteroid titer in the hemolymph. This suggests that the hemolymph ecdysteroid titer might be affected by DH secretion from SG in *O. fuscidentalis*. Therefore, our study aims to examine the sensitivity of diapausing larvae to *O. fuscidentalis* diapause hormone (Ompfu-DH) by observing morphological changes together with changes in the hemolymph ecdysteroid level, which is a key factor in the termination of larval diapause.

Materials and methods

Insects

O. fuscidentalis diapausing larvae were obtained from a bamboo forest in Maewang District Chiang Mai Province, Thailand in November 2014.

Injection of Ompfu-DH-like peptide

Based on the deduced amino acid sequence from cDNA, the twenty-four amino acid amidated peptide (Ompfu-DH-NH_2, VDDLKDEADRGASDRGTLWFGPRL-NH_2) from Suang et al., 2015 was synthesized by Bio Basic, Inc. (Canada) and stored at − 35 °C until use. The stock solution was diluted with distilled water to various concentrations (25, 50, 100 ng/5 μl). The larvae were injected with 5 μl aliquots (twenty larvae for each concentration) through a fine glass capillary. For the control, larvae were injected with distilled water. Control and experimental larvae were kept separately at 25 °C in continual darkness. Pupation was observed by pupal cuticle formation (Singtripop et al., 2000) within 30 days after injection and the percentage of pupation was calculated. In addition, the sensitivity of Ompfu-DH was examined by recording the duration time from the day of Ompfu-DH treatment to the day of pupal cuticle formation (Singtripop et al., 2002a).

Hemolymph collection

According to Singtripop et al. (2000), pupation in the bamboo borer could be categorized into 6 grades (G0–G5) of cuticle formation after hormone treatment. After injection of Ompfu-DH, larval hemolymph was collected by incision of the 2nd prolegs every 5 days until pupation occurred. The hemolymph (30 μl) was mixed with 270 μl of methanol and centrifuged at 10,000 × *g* for 5 min. The supernatant was transferred to a small test tube and dried in vacuo at room temperature. The residue was stored at − 20 °C (Singtripop et al., 1999) to measure the 20E concentration using enzyme-linked immunosorbent assay (ELISA).

Ecdysteroid analysis

Ecdysteroid was measured with the 20-hydroxyecdysone EIA kit (Cayman Co, Germany). This assay utilizes immobilized 20-hydroxyecdysone-specific antibodies and the ecdysone-acetylcholinesterase enzyme (AChE), which binds efficiently to ecdysone. This assay was performed in a 96-well microplate precoated with mouse antirabbit IgG. Samples were analyzed in triplicate. The ecdysteroid in each sample was compared to the 20-hydroxyecdysone standard curve and expressed as ng/ml in 20-hydroxyecdysone equivalents. The samples were diluted with EIA buffer and the colorimetric reaction was measured at 405 nm.

Results

Effect of Ompfu-DH on termination of larval diapause

After injecting 100 ng of synthetic Ompfu-DH into the diapausing larvae, they turned brown as described by Singtripop et al. (2000), which indicated that pupation had occurred. The effects of Ompfu-DH were further examined by injecting different concentrations of DH into diapausing larvae, which were then observed for 30 days. Two forms of pupation were observed in the bamboo borer after injection. Some larvae shed the old cuticle and formed complete pupae (Fig. 1A) and some larvae generated incomplete pupae (Fig. 1B). In the latter form of pupation, the larvae produced an additional tanned pupal cuticle beneath the old cuticle (Singtripop et al., 2000). When the diapausing larvae were injected with a lower concentration of Ompfu-DH, they eventually pupated. The lowest effective dose in this experiment was 25 ng/larvae.

The number of complete and incomplete pupae induced by Ompfu-DH is shown in Table 1. The percentage of pupation increased within 20 days, whereas only 5% of pupation was observed in larvae injected with 100 ng. Pupation continued when the period of observation was extended to 25 days. During this period, 25% of larval samples that were injected with 25 ng and 50 ng had pupated, while the number increased to 30% in larvae injected with 100 ng. Within 30 days of observation, almost half (45%) of all injected larvae had pupated (Fig. 2). Pupation was not observed in the control-diapausing larvae within 30 days.