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# An in vitro study on regulation of prothoracic gland activity in the early last-larval instar of the silkworm *Bombyx mori*

Akira Mizoguchi[a,*], Hiroshi Kataoka[b]

[a]*Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan*
[b]*Department of Integrated Biosciences, Graduate School of Frontier Science, The University of Tokyo, Kashiwanoha 5-1-5, Kashiwa, Chiba 277-8562, Japan*

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**Abstract**

The endocrine mechanisms that regulate prothoracic gland (PG) activity in early stages of final larval instar of the silkworm *Bombyx mori* were investigated using a newly developed long-term cultivation system of the gland. The PGs dissected from day-0 fifth instar larvae did not secrete detectable amounts of ecdysone for the first 24 h in culture but started secretion within the next 2 days. The amount of secreted ecdysone increased day by day. When day-0 PGs were co-cultivated with corpora allata, however, they remained inactive for at least 8 days. PGs dissected from 1-day younger larvae (day-3 fourth instar larvae) secreted ecdysone for the first 24 h but stopped secretion for the next 24 h, followed by recovery of ecdysone secretory activity. By contrast, PGs from day-1 fourth instar larvae remained active throughout a cultivation period without any sign of inactivation. However, when the same glands were exposed to a high titer of 20-hydroxyecdysone for the second 24 h in culture, they gradually lost their activity. These results indicate that PGs of fourth instar larvae are inactivated by ecdysteroid through a negative feedback mechanism and that thus inactivated PGs spontaneously recover ecdysone secretory activity in the early fifth instar unless inhibited by juvenile hormone.


*Keywords:* Prothoracic gland; In vitro culture; *Bombyx mori*; Juvenile hormone; Negative feedback

## 1. Introduction

Molting and metamorphosis of insects are induced by ecdysteroids secreted by the prothoracic glands (PGs). Therefore, the developmental schedule of insects is defined primarily by a temporal pattern of changes in ecdysteroid secretory activity of the PGs. However, the mechanisms regulating the changes in PG activity are not fully understood. Several factors are known to be involved in these mechanisms. The prothoracicotropic hormone (PTTH), which in Lepidoptera is produced by the brain and released from the corpora allata (CA), stimulates the PG to secrete ecdysteroids (for reviews, Bollenbacher and Granger, 1985; Ishizaki and Suzuki, 1994; Gilbert et al., 1996). Recently, developmental changes in PTTH titer in hemolymph have been investigated in the silkworm *Bombyx mori,* revealing a close relationship between the PTTH and ecdysteroid titers (Mizoguchi et al., 2001, 2002). Although these observations strongly supported a pivotal role of PTTH in regulating PG activity, some changes in PG activity, which were estimated by hemolymph ecdysteroid titer changes, could not be explained by the change in hemolymph PTTH titer. For example, immediately after ecdysis to the fifth instar, the PTTH titer increases to a low but significant level and maintains a similar level for several days. In contrast, the ecdysteroid titer is quite low for the first day after ecdysis and slowly but steady increases thereafter (Sakurai et al., 1998; Mizoguchi et al., unpublished observation). It has been reported in

*Corresponding author. Tel.: +81 52 78 95 039; fax: +81 52 78 92 511.
*E-mail address:* mizoguch@bio.nagoya-u.ac.jp (A. Mizoguchi).

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some Lepidoptera that injection or topical application of juvenile hormone (JH) or its analog to early final instar larvae delays initiation of larval–pupal transformation (Nijhout and Williams, 1974; Cymborowski and Stolarz, 1979; Akai and Kobayashi, 1971; Sakurai and Imokawa, 1988). In addition, Sakurai et al. (1989) demonstrated in *B. mori* that allatectomy on the first day of the fifth instar shortens the time between larval ecdysis and gut purge. These results suggest that JH plays an important role in regulating PG activity in the early stage of the fifth instar. However, it is still unclear whether JH acts on the PG directly or indirectly, because the effect of JH can be mediated by other hormone(s).

Analyses of the responses of PG in vitro to given regulatory factors are helpful for understanding their roles in the regulation of the PG in vivo. To our knowledge, no in vitro PG culture system has been reported that allows analyzing long-term effects of those factors, although a short-term incubation system of the PG has often been used in the assessment of PG activity (Bollenbacher et al., 1975; Sakurai, 1984; Okuda et al., 1985), determination of PTTH activity (Bollenbacher et al., 1979; Agui et al., 1979; Shirai et al., 1993) and analysis of PTTH signaling pathways (Smith et al., 1984; Gu et al., 1996). Hence, we first developed a method for long-term cultivation of the PG and then investigated the mechanisms by which the ecdysteroid secreting activity of the PG is regulated in early stages of the fifth instar. In this study, we show that ecdysteroids and JH but not PTTH are involved in regulating PG activity during these stages.

## 2. Materials and methods

### 2.1. Experimental animals

Males of a racial hybrid of *B. mori,* Kinshu × Showa, were used. Larvae were reared on an artificial diet "Silk Mate 2S" (Nihon Nosan Kogyo, Yokohama, Japan) at 25 °C under a photoperiod of LD12:12. Developmentally synchronous populations of the fourth and fifth (final) instars were obtained by collecting newly ecdysed larvae shortly after lights-on. This day was designated as day 0, with the lights-off time being the beginning of each day (0:00). Since ecdysis to the fourth instar occurred during the latter half of the photophase, the collected fourth instar larvae were 12–18-h old at the time of segregation (at 12:00 on day 0). Fourth instar larvae stopped feeding late on day 2 and ecdysed to the fifth instar on day 4 ( = day 0 of the fifth instar). Since ecdysis to the fifth instar occurred during the scotophase, the collected fifth instar larvae were 0–12-h old at the time of segregation.

### 2.2. Hormones

20-Hydroxyecdysone (20E) was purchased from Sigma. PTTH was expressed in bacteria and then purified (Ishibashi et al., 1994).

### 2.3. In vitro culture of PG

Dissections were carried out in insect saline (Jungreis et al., 1973) containing 5 mM sodium dimethyldithiocarbamate as a phenoloxidase inhibitor. Saline and culture medium (Grace's medium, pH 6.3, supplemented with 2% bovine serum albumin (BSA), 100 units/ml penicillin and 100 μg/ml streptomycin) were sterilized by filtration before use. Larvae were immersed in 70% ethanol for 2 min and then rinsed twice with sterile distilled water. Under saline, the thorax of larvae was cut open from the ventral side and a small piece (approximately 2 mm × 2 mm) of body wall including the PG was cut out with Vannas-style spring scissors. Since the anterior end of the gland was attached to some tissue within the head, this end was pulled out with forceps. This piece of body wall contained the integument, trachea, muscle and fat body. Salivary gland, thoracic ganglion and suboesophageal gland were carefully removed, if contained. The piece of body wall including the PG (simply called PG hereafter) was rinsed twice with 0.75 ml of saline and then cultivated in 100 μl of culture medium in wells of a 96-well cell culture plate (Falcon, 3075). The plate was maintained at 25 °C under a photoperiod of LD12:12 and an oxygen partial pressure ($PO_2$) of 40–50%. To keep this high $PO_2$, the culture plate was placed in a gas-exchangeable desiccator (300 × 280 × 290 mm) with a water-filled humidity pan and an oxygen monitor inside. Oxygen was supplied to the desiccator from a cylinder to bring an initial $PO_2$ of approximately 50% and then the stopcocks closed. The $PO_2$ decreased slowly reaching nearly 40% after 24 h. The culture medium was renewed every 24 h, at 3 h after lights-on, by removing all the old medium from and refilling fresh medium to the culture. The collected medium was stored at −30 °C until ecdysteroid determination.

### 2.4. Ecdysteroid determination

The concentration of edysteroids in culture medium was determined by radioimmunoassay. Culture supernatant was mixed with 1.5 volumes of assay buffer (50 mM Tris-HCI buffer, pH 7.6, containing 0.2% BSA), and 50 μl of this mixture was pipetted into a miniscintillation vial, followed by mixing with 50 μl of [23, 24-$^3$H] ecdysone (ca. 10,000 cpm, New England Nuclear Co.) in assay buffer, 50 μl of 1:4000 diluted anti-ecdysone rabbit antiserum and 50 μl of scintillation proximity assay reagent, which contains anti-rabbit Ig