Bull. Inst. Zool., Academia Sinica 27(1): 1-5 (1988)

EFFECT OF ECDYSTERONE ON RED FLUORESCENT PROTEIN INTENSITY IN THE SILKWORM, *BOMBYX MORI* L.

ROGER F. HOU, C. S. CHIU[1] and MEI-LIN TSAI[2]

Department of Entomology, National Chung Hsing University, Taichung, Taiwan, 40227, Republic of China

(Accepted September 14, 1987)

Roger F. Hou, C. S. Chiu and Mei-Lin Tsai (1987) Effect of ecdysterone on red fluorescent protein intensity in the silkworm, *Bombyx mori* L. *Bull. Inst. Zool., Academia Sinica* 27(1): 1-5. The variation in antiviral red fluorescent protein (RFP) intensity purified from midgut of the silkworm, *Bombyx mori*, was studied using thoracic ligation and exogenous administration of ecdysterone. The 4th instar larvae had less RFP intensity after thoracic ligation from 24 to 44 hr post-ecdysis, but the intensity was greatly increased by injecting ecdysterone especially within 24-36 hr after ecdysis. The RPF intensity in ligated 4th instar larvae could be compensated by injecting ecdysterone at 0.4 μg/μl. Therefore, it is suggested that the RFP synthesis in midgut could possibly be regulated by a cephalic factor(s) and ecdysteroids.

Key words: *Bombyx mori*, Silkworm, BmNPV, RFP, Ecdysterone.

The silkworm, *Bombyx mori*, contains antiviral substances in its gut juice (Aizawa, 1962). A red flourescent protein (RFP) was isolated from silkworm gut juice and to be able to inhibit *B. mori* nuclear polyhedrosis virus (BmNPV) infection (Muka *et al.*, 1969; Hayashiya *et at.*, 1971). The antiviral activity of the RFP was proposed to be due to the enzymatic activity of phospholipase C (Hou and Chiu, 1986). Liu and Hou (1985) has indicated reduction in larval mortality caused by BmNPV due to administration of an ecdysteroid-containing agent when given before viral inoculation. There is a peak of ecdysone titer immediately before the 3rd ecdysis in *B. mori* followed by a highest RFP intensity during the 4th ecdysis (Hayashiya, 1975; Calvez *et al.*, 1976; Tojo *et al.*, 1981). Furthermore, ecdysteroids may be stimulative to midgut protein syntheses in lepidopterous larvae (Doane, 1973). It is thus reasonable to speculate that the RFP formation is regulated by ecdysteroids in silkworm midgut. This paper presents evidence that RFP intensity is affected by thoracic ligation and ecdysterone injection.

MATERIALS AND METHODS

Insects

The silkworm, *B. mori*, was a hybrid of (Kuo×Fu) X (Nung×Feng) raised by the Taiwan Sericultural Improvement Station, Miaoli. The eggs were incubated at 25°C, 75% R. H. for 10-12 days. The newly hatched larvae were reared on mulberry leaves until the 4th instar.

Purification and measurement of RFP

The purification of RFP was carried not

1. Department of Applied Zoology, Taiwan Agricultural Research Institute, Wufeng, Taichung, Taiwan.
2. Taiwan Sericultural Improvement Station, Miaoli, Taiwan.

according to the procedures modified from Hayashiya *et al.* (1968), but the ion exchange step was omitted since Chiu (1983) found that one-step chromatography using Sephadex G-75 filtration only is as efficient as two-step chromatography. The fluorescence strength of the purified RFP was read as emission at 660 nm after excitation at 550 nm with a fluorescence spectrophotometer (Perkin-Elmer 2000). Each fluorescence strength measurement was calculated on the basis of unit larval weight. Comparison of RFP intensity among various treatments was based on the highest fluorescence strength as 100%.

Thoracic ligation and ecdysterone treatment

The 4th instar larvae were ligated with nylon threads around the thoracic segment between the 1st and 2nd pairs of prolegs at different ages. Fifty larvae were ligated in each treatment. The midgut was dissected out through the dorsal side on day 3 post-ligation. The RFP was extracted and purified from the dissected midguts, and that purified from the larvae without thoracic ligation served as a control.

For ecdysterone administration, the 4th instar larvae of various ages were injected with ecdysterone (Sigma Chemical Co., St. Louis, Mo., USA, ecdysterone was dissolved in ethanol at a final concentration of 0.4 μg/μl) through the abdominal intersegmental membrane by using an automatic microsyringe. The midgut RFP was purified 3 days after injection. In the other series of experiments, the 4th instar larvae 60-66 hr after ecdysis were ligated and then injected with 0.4 μg/μl ecdysterone 24 hr after ligation; RFP was then purified from midguts after incubating for 2 more days.

RESULTS

The 4th instar larvae 24-96 hr after ecdysis were ligated through their thoracic segments. The RFP produced by the ligated larvae was purified, and its intensity was measured. The RFP intensity of ligated larvae 24-44 hr post-ecdysis was significantly

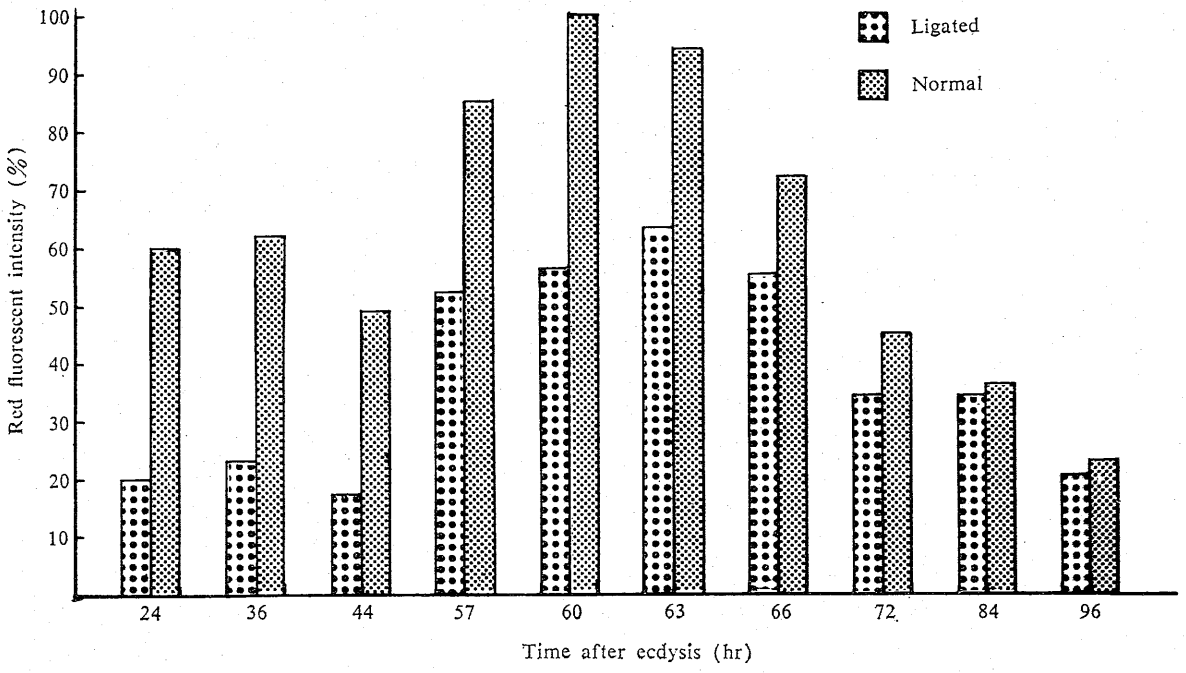

Fig. 1. Red fluorescent intensity of the purified RFP from 4th instar *B. mori* after ligation at various larval ages.

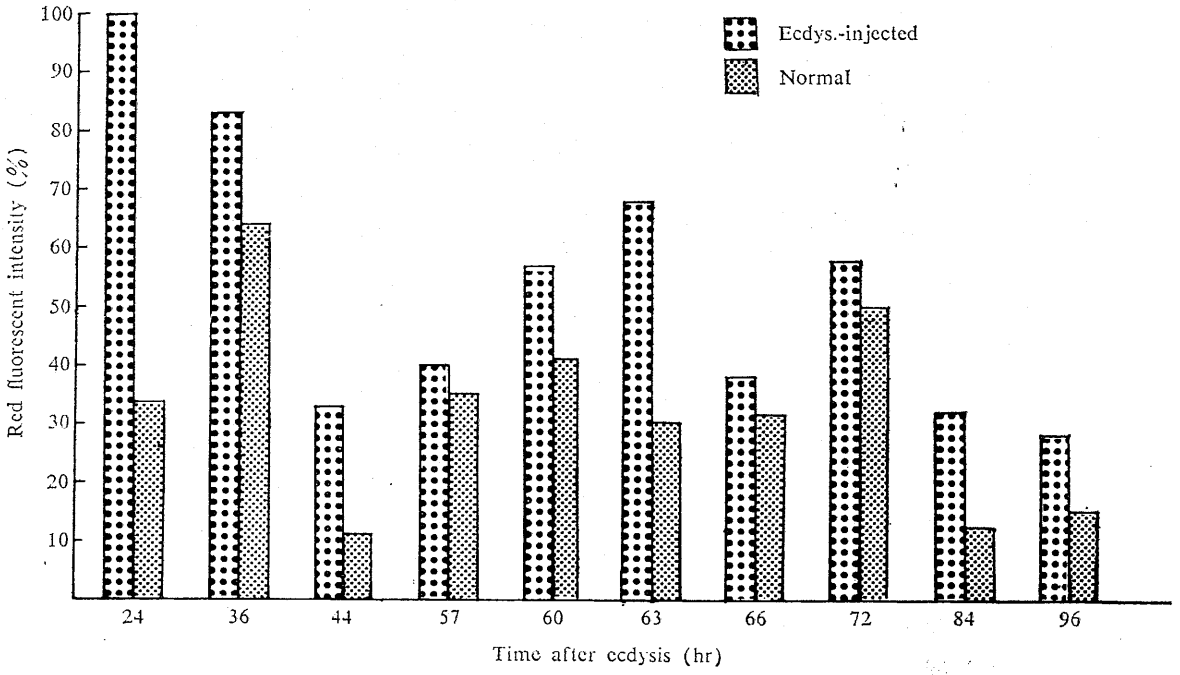

Fig. 2. Red fluorescent intensity of the purified RFP from 4th instar *B. mori* after injection of ecdysterone at various larval ages.

lower than the normals, the difference being almost 3 fold. However the intensity between the normal and the treated insects became closer starting from 66 hr and reached almost the same level by 84 hr after ligation. The lowest RFP intensity was obtained from the ligation at 44 hr post-ecdysis, while the production of RFP was not obviously deterred if ligated 72 hr after ecdysis (Fig. 1).

The larvae were injected with ecdysterone from 24 hr to 96 hr after ecdysis. Fig. 2 shows stimulation of RFP production by exogenous administration of ecdysterone. The highest RFP intensity resulted from injection of ecdysterone by 24–36 hr post-ecdysis. In comparison of all treatments, the ecdysterone-injected larvae produced more RFP than the uninjected larvae, while the larvae injected with ethanol solution only had RFP strength similar to the normal insects. The 4th instar larvae were ligated through thorax 60, 63 or 66 hr after ecdysis, and were injected with ecdysterone at 0.4 $\mu g/\mu l$ one day after ligation. The RFP contents of these insects were determined 2 days after ecdysterone injection. Fig. 3 shows that the normal larvae contained higher RFP intensity than the injected insects in 60- and 63-hr-ligated treatments,

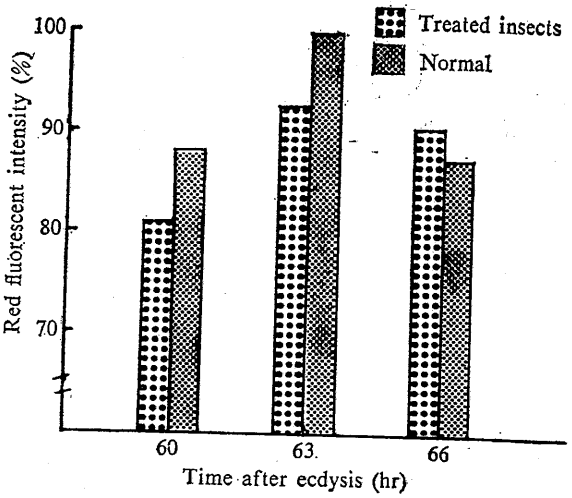

Fig. 3. Red fluorescent intensity of the purified RFP from the ligated 4th instar *B. mori* after injection of ecdysterone at 60–66 hr post-ecdysis.

but the intensity was slightly higher in ecdysterone-injected larvae than in the normals if treated at 66 hr post-ecdysis. In contrast, the ligated larvae without injecting ecdysterone at the same age produced much less RFP (Fig. 1). It is thus hypothesized that ecdysterone functions as an effector to rescue the reduction in RFP formation due to thoracic ligation and that a cephalic factor(s) is essential for its production.

DISCUSSION

Injection of exogenous ecdysteroids may reduce larval mortality of *Heliothis virescens* inoculated with nuclear polyhedrosis virus (NPV) (Keeley and Vinson, 1975). Although the hormonal inhibition of viral injection in insects is still unclear, the antiviral activity enhanced by insect hormones could possibly be explained by the following hypotheses: (1) hormones could directly reduce the viral replication in host cells; (2) hormones might regulate the cellular metabolism to defend the viral infection in susceptible cells; (3) hormones could stimulate the production of antiviral substance(s) in host cells. The hormonal interaction between insect hosts and their parasites has been elucidated by *Beckage* (1985). It is not known if insect hormones might activate antiviral activity of host cells in the same manner as parasites do. However, our results seem to support the 3rd hypothesis mentioned above.

The RFP synthesized in silkworm midgut is effective in suppressing BmNPV infection (Hayashiya, 1978; Hou and Chiu, 1986). Hence, factors stimulating RFP formation should be capable of enhancing antiviral activity. Liu and Hou (1985) reported reduction in larval mortality of *B. mori* by administrating an ecdysteroid-containing agent before inoculating with BmNPV. They proposed that ecdysteroids could strengthen antiviral activity in silkworms. The present results suggst that the production of RFP is regulated by a cephalic factor(s) as revealed by thoracic ligation, and that the exogenous administration of ecdysteroids could increase RFP intensity and compensate for the reduction in the intensity resulting from thoracic ligation. Regulation of midgut protein synthesis by ecdysone is possible in lepidopterans (Doane, 1973) and the fact that ecdysone synthesis in prothoracic glands is activated by the prothoracicotropic hormone (PTTH) released by corpora allata has been proved in insects (Bollenbacher and Granger, 1985). It is thus not surprising to note that RFP formation is reduced after thoracic ligation. However, this synthetic activity can be rescued by injecting exogenous ecdysterone into silkworms as shown in the present study. Therefore, the antiviral RFP in silkworm midgut could possibly be synthesized under the hormonal control of ecdysteroids.

Acknowledgements: This study was supported by a research grant No. NSC73-0649-B005-02 from the National Science Council, ROC. Thanks are extended to the Taiwan Sericultural Improvement Station for suppying silkworms.

REFERENCES

AIZAWA, K. (1962) Antiviral substance in the gut-juice of the silkworm, *Bombyx mori*. *J. Insect Pathol.* **4**: 72-76.

BECKAGE, N. E. (1985) Endocrine interactions between endoparasitic insects and their hosts *Ann. Rev. Entomol.* **30**: 371-413.

BOLLENBACHER, W. E. and N. A. GRANGER (1985) Endocrinology of the prothoracicotropic hormone. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (G. A. KERKUT and L. I. GILBERT, eds.). Pergamon Press, Oxford. **7**: 109-151.

CALVEZ, B., M. HIRN and M. DE REGGI (1976) Ecdysone changes in the haemolymph of two silkworms (*Bombyx mori* and *Philosamia cynthia*) during larval and pupal development. *FEBS Letters* **71**: 57-61.

CHIU, C. S. (1983) Studies on the red fluorescent protein (RFP) in the gut juice of the silkworm, *Bombyx mori* L. M. S. Thesis, National Chung Hsing University, Taichung, Taiwan, R. O. C. 53 pp. (In Chinese)

DOANE, W. W. (1973) Role of hormones in insect development. In *Developmental Systems: Insects* (S. J. COUNCE and C. M. WADDINGTON, eds.). Academic press, London. **2**: 291-497.

HAYASHIYA, K. (1975) Artificial diets and fresh mulberry leaves for silkworms. In *Methods for Artificial Rearing of the Silkworm* (Y. HAMAMURA, ed.). Misuzu Shobo, Tokyo. pp. 71-107. (In Japanese)

HAYASHIYA, K. (1978) Red fluorescent protein in the digestive juice of the silkworm larvae fed on host-plant mulberry leaves. *Entomologia exp. appl.* **24**: 228-236.

HAYASHIYA, K., J. NISHIDA and F. F. MATSUBARA (1968) Inactivation of nuclear polyhedrosis virus in the digestive juice of silkworm larvae, *Bombyx mori* L. I. Comparison of anti-viral activities in the digestive juice of larvae reared on natural and artificial diets. *Jpn. J. app. Ent. Zool.* **12**: 189-193. (In Japanese)

HAYASHIYA, K., F. MATSUBARA, J. NISHIDA and F. KAWAMOTO (1971) Comparative experiments with the silkworm larvae reared on mulberry leaves and artificial diets. I. Comparison of antiviral activities in the digestive juice of larvae reared on natural and artificial diets. *Bull. Faculty of Textile Sci.* **6**: 87-100. (In Japanese)

HOU, R. F. and C. S. CHIU (1986) Antiviral proteins in midgut of the silkworm, *Bombyx moir*, fed on different food sources. *Entomologia exp. appl.* **42**: 3-8.

KEELEY, L. L. and S. B. VINSON (1975) β-ecdysone effects on the development of nucleopolyhedrosis in *Heliothis* spp. *J. Invertebr. Pathol.* **20**: 121-123.

LIU, H. S. and R. F. HOU (1985) Studies on inhibition of infection with nuclear polyhedrosis virus by an ecdysteroid-containing agent in the sikworm *Bombyx mori* L. *Chinese J. Entomol.* **5**: 23-30.

MUKAI, J., J. INOUYE and S. AKUNE (1969) A red fluorescent protein from silkworm. *Agr. Biol. Chem.* **33**: 125-127.

TOJO, S., K. KIGUCHI and S. KIMURA (1981) Hormonal control of storage protein synthesis and uptake by the fat body in the fat body in the silkworm, *Bombyx mori*. *J. Insect Physiol.* **27**: 491-497.

昆蟲脫皮素對家蠶紅色螢光蛋白質强度之效應

侯豐男　邱紀松　蔡美玲

由家蠶中腸所純化之抗病毒紅色螢光蛋白質强度的變異，以胸部結紮及施加脫皮素研究之。四齡幼蟲自蛻皮後 24～44 小時施行胸部結紮，則紅色螢光蛋白質强度降低，但在蛻皮 24～44 小時注入脫皮素使其强度增加。胸部結紮之四齡幼蟲之紅色螢光蛋白質强度可由注入 0.4 μg/μl 之脫皮素所補償。因此，本文建議家蠶中腸之紅色螢光蛋白質合成作用可能受頭部因素與脫皮素之調制。