Appl. Ent. Zool. **23** (3): 213–219 (1988)

Ovarian Prothoracicotropic Hormone Activity in the Silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)

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(Received April 22, 1987)

Prothoracicotropic hormone (PTTH) activity assayed in brainless pupae of *Samia cynthia ricini* is present in the mature eggs of the silkworm, *Bombyx mori*. Comparable activity was also found in eggs which had matured in brainless moths and in isolated abdomens. No significant differences in chemical components were shown in the eggs developed at three different hormonal milieus. It is possible to conclude that the ovarian PTTH is synthesized during egg maturation.

INTRODUCTION

Prothoracicotropic hormone (PTTH) is a neuropeptide hormone that activates the prothoracic glands in which ecdysone synthesis occurs (BOLLENBACHER and GRANGER, 1985).

In *Bombyx*, two distinct forms of PTTHs are known to be present in adult heads or pupal brains: one activates the *Bombyx* prothoracic glands (PTTH-B), and the other (PTTH-S) stimulates the ecdysone synthesis in *Samia* prothoracic glands (AIZONO et al., 1986; ISHIZAKI and SUZUKI, 1984; NAGASAWA et al., 1984; SUZUKI, 1986).

Four distinct neurohormones (PTTHs, eclosion hormone, diapause hormone) are also present in the embryonating eggs of the silkworm, *Bombyx mori* (CHEN et al., 1986, 1987; FUGO et al., 1985, 1987). A previous paper (FUGO et al., 1987) clearly demonstrated that PTTH-S activity was detected in the extracts of developing ovaries. It remains to be resolved whether this ovarian PTTH-S activity was from the host brain or was synthesized *in situ* by the ovary itself. In this paper, the authors deal with the effects of brains and molting hormone (20-hydroxyecdysone) on the accumulation of PTTH-S in the ovaries.

MATERIALS AND METHODS

Animals. Larvae of *Bombyx mori* (Japanese No. 122 × Chinese No. 115) were reared on mulberry leaves at 25±2°C under natural photoperiodic conditions.

Larvae of *Samia cynthia ricini* were reared on *Ailanthus glandulosa* leaves at 25±2°C. After pupation, their brains were extirpated and the brainless *Samia* pupae were used for PTTH-S bioassay.

Surgical operations. Isolated abdomen. Two to three hours before pupation, female pharate pupae of the *Bombyx* were ligatured between thorax and abdomen with fine cotton thread, and the part anterior to the ligation was cut off. The isolated abdomens were kept at 25±1°C until use. Ten days after ligation, 20-hydroxyecdysone (2.0 μg/abdomen) was injected into the isolated abdomens and they were maintained under the same condition.

Brain extirpation. The brain was extirpated from a female pupa of *Bombyx* at 10 hr after pupation. The wound was sealed with melted paraffin. The brainless pupae were maintained at 25±1°C. In some experiments, the brainless pupae received an injection of PTTH-B (2 units/20 μl/animal) 2 days after the brain extirpation and were kept at 25±1°C.

Preparation of the mature eggs. On 1 day before adult eclosion or just after eclosion, normal and brainless moths were dissected in cold saline solution (0.8% NaCl) and the ovaries were taken out. Mature eggs were collected from the isolated abdomens 14 days after 20-hydroxyecdysone treatment. The ovaries were placed on gauze and rubbed gently to separate the eggs from the oviducts. The eggs were rinsed with cold water three times and the mature eggs with chorion were collected. The extent of ovarian development was estimated by measuring the ovary weight and the number of fully matured eggs.

Extraction and partial purification of prothoracicotropic hormones. The extraction and partial purification of the PTTH-S from ovaries was carried out in a cold room (4°C) as described previously (FUGO et al., 1987). PTTH-B was extracted and partially purified from the adult heads of the silkworm by the method described by MATSUO et al. (1985). Specific activity of this hormone was about 15 μg/*Bombyx* unit.

20-Hydroxyecdysone was purchased from Rhoto Pharmaceutical Co. Ltd. (Osaka, Japan) and dissolved in distilled water (1 mg/ml).

Bioassay for prothoracicotropic hormone-S. Samia brainless pupae were used for the determination of PTTH-S activity (ISHIZAKI and ICHIKAWA, 1967). Forty days after extirpation of the brain, pupae which showed no signs of pupal-adult development were used for bioassay. Partially purified material from ovarian eggs was dissolved in 0.1 M Tris-HCl buffer (pH 7.4, two pairs of ovary equiv./20 μl) and an indicated dose of the material was injected into a brainless pupa (20 μl/pupa). The injected pupae were kept at 25±1°C. Arrested adult development of *Samia* brainless pupae was reinstated and adult eclosion occurred 18 to 22 days after injection if the injected material had PTTH activity. One unit of the PTTH-S activity represented the minimal dose of PTTH inducing adult development in more than 50% of the assayed insects.

Measurement of lipids, proteins and glycogen. In order to measure the component content of the eggs, mature eggs with chorion were prepared from the adult ovaries.

Lipids were extracted from the eggs with chloroform/methanol (3: 1, v/v). After washing with 0.5 M NaCl solution, the chloroform layer was evaporated and the residue weighed.

Egg proteins were extracted with 0.4 M KCl in 50 mM phosphate buffer (pH 6.2) and the samples were applied on a 5% polyacrylamide gel (pH 8.4). Polyacrylamide gel electrophoresis was done according to the method of DAVIS (1964). Protein content was determined by the method of LOWRY et al. (1951) with bovine serum albumin as a standard.

For extraction of the glycogen, fresh eggs were homogenized in cold water and homogenates were passed through three layers of gauze to remove the chorions. The homogenates were then digested in 30% KOH at 100°C for 30 min, and 4 volumes of ethanol were added to precipitate the glycogen. Glycogen was determined with the phenol-sulphuric acid method (DUBOIS et al., 1956) with glucose as a standard.

RESULTS AND DISCUSSION

In a previous paper (FUGO et al., 1987) the authors reported that PTTH-S activity was present in extracts of the developing ovaries of *Bombyx mori*. In the present experiment, the authors succeeded in extracting the material eliciting adult development of brainless *Samia* pupae from the eggs which had matured in normal moths, in isolated abdomens and in brainless moths (Fig. 1, Table 2).

Figure 1 shows dose-response curves using PTTH-S obtained from eggs which developed under the three different conditions of pupal-adult development. The minimal amount needed to induce adult development in more than 50% of the brainless *Samia* pupae was equivalent to the material from about 0.75 pairs of ovaries in normal moths (Fig. 1). Since a female moth laid 500 to 600 eggs, 0.0022–0.003 units of PTTH-S was present in one egg of *Bombyx*. The authors earlier demonstrated that the amount of the hormone in developing ovaries (1 day before eclosion) was 0.005 to 0.007 units/egg (FUGO et al., 1987). The yield of the hormone extracted in this experiment was lower than that of the previous study, and the reasons for this discrepancy are considered to be due to lower sensitivity of the brainless *Samia* pupae to PTTH-S and to the use of different races for extraction of PTTH-S.

Male pupae of a specific racial hybrid of *Bombyx mori* (Japanese No. 122 × Chinese No. 115) have been used for bioassay of the PTTH-B (KOBAYASHI, 1955; ISHIZAKI et al., 1983; MATSUO et al., 1985; YAMAZAKI and KOBAYASHI, 1969), because the female brainless animals initiated the pupal—adult development spontaneously even if extirpa-

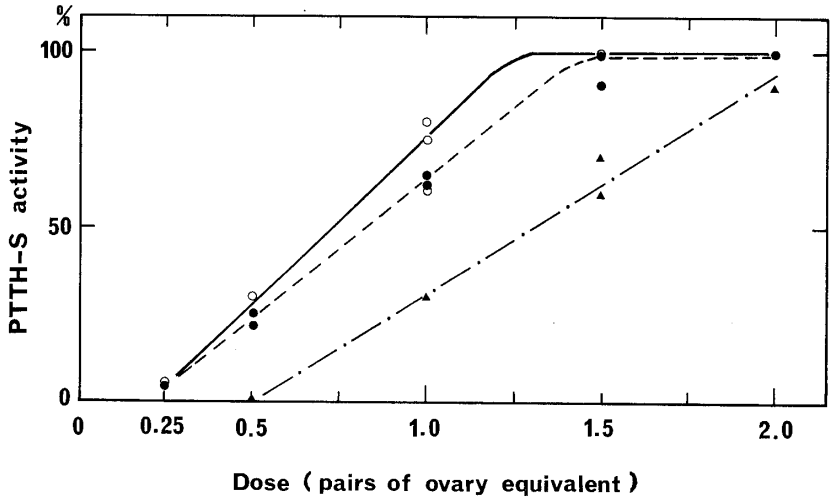

Fig. 1. Dose-response curves of prothoracicotropic hormone (PTTH) in the *Samia* assay. The PTTH was prepared from the ovaries developing in normal moths (○), in brainless moths (●) and in isolated abdomens (▲) of *Bombyx mori*.

Table 1. Ovary development in normal moths, in brainless moths and in isolated abdomens of the silkworm, *Bombyx mori*

Ovary source[1)]	Wet weight of ovaries (mg/animal)	No. of maturing eggs per animal
Normal moths[2)]	452.3±6.4 (n=10)	563.2±19.6 (n=10)
Brainless moths[3)]	455.3±5.4 (n=10)	570.0±20.6 (n=10)
Isolated abdomens[4)]	273.2±8.2 (n=15)	281.5±11.9 (n=14)

1) Ovaries in normal moths or brainless moths were taken out just after eclosion or one day before eclosion.
2) Controls had a sham operation in place of brain extirpation.
3) The brain was extirpated 10 hr after pupation.
4) Isolated abdomens were prepared about 2 to 3 hr before pupation and were administered 2 μg of 20-hydroxyecdysone 10 days after ligation. Ovaries were pulled out 14 days after the injection of 20-hydroxyecdysone.

Table 2. PTTH-S activities and some components of the eggs maturing in normal moths, in brainless moths and in isolated abdomens

Egg source	PTTH-S activity (units/g)	Lipid (mg/g)	Glycogen (mg/g)	Protein (mg/g)
Normal moths	5–7	86.3±2.6	27.2±1.5	104.4±3.8
Brainless moths	5–7	85.4±1.3	26.9±2.1	105.4±0.9
Isolated abdomens	6–8	84.2±5.8	26.9±2.3	107.0±3.6

PTTH-S activity was determined by *Samia* assay. Other details as in Table 1 and text.

tion of the brain was carried out just after pupation (ISHIZAKI et al., 1983). In the present case, eclosion of the brainless female moths occurred 11 to 13 days after the operation, indicating a sufficient amount of PTTH-B for pupal-adult development had already been secreted in the female. In these animals, ovarian development and the number of eggs developing in the brainless moths was similar to that of normal moths (Table 1). The titer of the PTTH-S in those eggs was almost the same as that in normal moths (Fig. 1, Table 2).

The ovaries developed in the isolated abdomens which had received 20-hydroxyecdysone 10 days after ligation also had the PTTH-S activity in their extracts (Fig. 1, Table 2). The hormonal titer in the mature eggs of the isolated abdomens was lower than that of normal animals and of brainless moths (Fig. 1). Since the volume of the body cavity was reduced by the ligation, the mass gain of ovaries developed in the isolated abdomens seemed to be considerably suppressed (Table 1). However, the amount of PTTH-S involved in one gram of eggs from those three ovaries was almost equal (Table 2).

As shown in Table 2, there was no difference in PTTH-S content in ovarian eggs grown in the three different hormonal milieus. Lipids, glycogen and proteins were accumulated during the egg maturation. Surgical operations of extirpation and transplantation of the brain or suboesophageal ganglion are well known to result in a change of voltism in this insect, and the contents of glycogen and lipids in the eggs are also changed by these operations (see for review YAMASHITA and HASEGAWA, 1985). The chemical composition of the mature eggs was investigated in the present study.

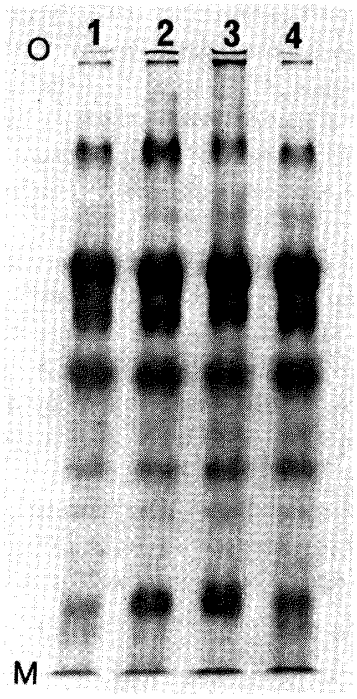

Fig. 2. Polyacrylamide gel electrophoresis of egg proteins. The eggs (800 mg) were homogenized with 4 ml of 0.4 M KCl in 50 mM phosphate buffer (pH 6.2). After centrifugation at 7,000 rpm (10 min, 4°C), 50 μl of glycerol-BPB solution (60% glycerol–0.002% bromophenol blue) was added to 30 μl of the supernatant. Ten μl of the mixture was applied on a 5% polyacrylamide gel: (1) normal eggs, (2) eggs from brainless moths, (3) eggs from isolated abdomens and (4) eggs from PTTH-B injected brainless moths. O: origin, M: marker front.

Figure 2 shows the polyacrylamide gel electrophoresis pattern of the egg proteins. No difference in pattern was observed from matured eggs with chorion collected from adult ovaries which had developed in the different hosts. PTTH-B injection into the brainless pupae also had no effect on the composition of the egg proteins (Fig. 2), nor was any influence observed of the brain extirpation or ligation on the contents of lipids, glycogen and proteins of these eggs (Table 2). Accordingly, eggs maturing in brainless moths or in isolated abdomens were in no way inferior to normal eggs in their major biochemical components.

All these results indicated that PTTH-S activity is present in the mature eggs in ovaries. This hormonal activity was also observed in embryonating and diapausing eggs (Fugo et al., 1987). At the early stage of embryogenesis in the silkworm, the PTTH-S activity was low but the titer abruptly increased after the differentiation of the central nervous system (Fugo et al., 1987). Results of the present study strongly suggest that PTTH-S activity exists in the yolk, but that in the late stages of embryogenesis the hormone is synthesized by the embryo itself.

The origin of the ovarian PTTH-S activity is still unknown. Based on the data shown in Fig. 1 and Table 2, about 1.3 units of PTTH-S are present in each pair of ovaries. About 8–10 units of PTTH-S are present in an adult silkworm head (Ishizaki and Suzuki, 1984; Chen et al., 1986). Overwhelmingly, the PTTH-S content is higher in the brain (head) than in the ovaries. A slight possibility remains that the PTTH-S present in ovarian eggs is derived from the brain of the mother. However, it is more probable that PTTH-S activity found in the ovaries is synthesized *in situ* during egg maturation, since the eggs developed in the brainless female moths or in the isolated abdomens have a PTTH-S activity almost equal in amount to that in normal moths (Table 2). To clarify the source of the ovarian PTTH-S activity, examination of PTTH-S activity in eggs cultured *in vitro* is necessary.

The role of PTTH-S on the development and growth of the silkworm, *Bombyx mori*, is not yet clear. Nishiitsutsuji-Uwo and Nishimura (1975) demonstrated that the thorax and abdomen of developing female *Bombyx* pupae contained a large quantity of PTTH. Comparison of the PTTH titer in normal moths with that in castrated female moths suggested that the accumulation of PTTH-S in the young female pupal bodies is consumed by the egg maturation and/or yolk deposition processes (Nishiitsutsuji-Uwo and Nishimura, 1975). On the other hand, Ishizaki (1969) reported that the PTTH activity was found only in the female thorax-abdomens during adult development and that it reached maximum quantity at the time of adult eclosion. This accumulated activity was most likely derived from the ovaries. Thus it is considered that the PTTH-S may somehow function in egg development. However, PTTH-S seems to have no gonadotropic action in the isolated abdomens (Fugo, unpublished data) and the injection of PTTH-S failed to induce the development of larval ovaries in male brainless pupae of the silkworm, *Bombyx mori* (Fugo and Segawa, 1987).

The prothoracicotropic function of PTTH-S in *Samia* has been clearly demonstrated (Ishizaki and Suzuki, 1984; Nagasawa et al., 1984). However, what is the physiological function or purpose of PTTH-S in *Bombyx*? The answer to this question continues to be eagerly sought.

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