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Molecular characterization and expression of prothoracicotropic hormone during development and pupal diapause in the cotton bollworm, *Helicoverpa armigera*

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Abstract

Using a strategy of rapid amplification of cDNA ends, the cDNA encoding prothoracicotropic hormone (PTTH) was cloned from the brain of *Helicoverpa armigera* (Hearm). The Hearm-PTTH cDNA contains an open reading frame encoding a 226-amino acid preprohormone, which shows high identity with the closely related noctuid moths, *Helicoverpa zea* (98%) and *Heliothis virescens* (94%), and low identity with five species of Bombycoidea: *Bombyx mori* (57%), *Manduca sexta* (55%), *Hyalophora cecropia* (52%), *Samia cynthia ricini* (49%) and *Antheraea peryni* (48%). Hearm-PTTH cDNA shares important structural characterization known from other PTTHs, such as seven cysteine residues, proteolytic cleavage site, glycosylation site, and hydrophobic regions within the mature peptides. Northern blot analysis indicated a 0.9 kb transcript present only in the brain. Using the more sensitive technique of RT-PCR, PTTH mRNA was also detected in the subesophageal ganglion, thoracic ganglion, abdominal ganglion, midgut and fat body. During the pupal stage, PTTH mRNA in the brain remained at a constant high level in nondiapausing individuals, was low in diapausing pupae, but increased again at diapause termination. The PTTH protein was detected only in the brain by Western blot analysis. Immunocytochemical results revealed that Hearm-PTTH is localized in two pairs of dorsolateral neurosecretory cells within the brain. Recombinant Hearm-PTTH was successfully expressed in *E. coli*, and purified recombinant-PTTH was effective in breaking pupal diapause. The results are consistent with a role for PTTH in the regulation of diapause in this species.
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1. Introduction

Prothoracicotropic hormone (PTTH) is a brain neuropeptide responsible for the stimulation of the prothoracic glands (PGs) to produce ecdysteroids, thereby playing a central role in the endocrine network controlling insect growth, molting, metamorphosis and diapause (Denlinger, 1985; Ishizaki and Suzuki, 1994; Gilbert et al., 2002). PTTH was first purified from the silkworm, *Bombyx mori* (Bomor): Bomor-PTTH is a 30 kDa peptide consisting of two identical subunits linked by a disulfide bond as demonstrated by peptide sequencing and cDNA cloning studies (Kawakami et al., 1990; Kataoka et al., 1991). Using molecular techniques, PTTH has now been identified from *Samia cynthia ricini* (Ishizaki and Suzuki, 1994), *Antheraea pernyi* (Sauman and Reppert, 1996), *Hylophora cecropia* (Sehnal et al., 2002), *Manduca sexta* (Shionoya et al., 2003), *Heliothis virescens* (Xu and Denlinger, 2003) and *Helicoverpa zea* (Xu et al., 2003).

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Pupal diapause is thought to be the consequence of a shut-down in PTTH synthesis and/or release and the subsequent failure of the PGs to synthesize the ecdysteroids needed to promote continuous development (Denlinger, 1985). Thus far, most evidence that PTTH regulates insect diapause is from members of the Lepidoptera superfamily, the Bombycoidea: *A. pernyi* (Williams and Adkisson, 1964a, b; Sauman and Reppert, 1996), *H. cecropia* (Williams, 1947; Sehnal et al., 1981) and *M. sexta* (Shionoya et al., 2003). Recently, PTTH molecules from two noctuid moths, *H. virescens* and *H. zea*, were cloned, and our preliminary experiments indicated that expression of the *H. virescens* PTTH gene was low during diapause, a result consistent with the prevailing hypothesis that a shut-down in PTTH production is responsible for diapause induction in pupae (Xu and Denlinger, 2003). But, the pupal diapause in our colonies of *H. virescens* and *H. zea* can be rather easily terminated; thus these species are not ideal for rigorously investigating the effect of PTTH on diapause termination. Therefore, in our previous experiments we only investigated changes of PTTH in *H. virescens* and *H. zea* at the level of the mRNA (Xu and Denlinger, 2003; Xu et al., 2003). The pupal diapause of *Helicoverpa armigera* is more robust and thus it was used in our current experiments.

Recently, diapause hormone (DH) has also emerged as a potential player in the termination of diapause in *H. armigera* (Zhang et al., 2004a) and *H. virescens* (Xu and Denlinger, 2003). In both of these species the gene encoding DH is down-regulated in diapausing pupae and up-regulated in nondiapausing pupae, and an injection of DH prompts diapause termination. Further studies with *H. armigera* show that DH breaks diapause by activating the PG to synthesize ecdysteroids (Zhang et al., 2004b). If DH can break diapause, what is the function of PTTH in diapause termination?

To address the function of PTTH in the diapause of *H. armigera*, it was necessary to first characterize the PTTH molecule in this species. In the present study, we report the molecular characterization of Hearm-PTTH cDNA from the brain using degenerate primers deduced from known PTTH cDNAs. Expression analysis reveals that the Hearm-PTTH gene is predominantly expressed in the brain, and immunocytochemical evidence indicates that, within the brain, the Hearm-PTTH protein is localized in two pairs of dorsolateral neurosecretory cells. The homodimeric PTTH (30 kDa) and PTTH subunit (15 kDa) are detected only in the brain by Western blot analysis. We will also show that the recombinant PTTH (re-PTTH) expressed in *E. coli* is able to terminate diapause in pupae of *H. armigera*. Thus, we demonstrate that two distinctly different neuropeptides, DH and PTTH, are fully capable of breaking diapause in this species.

2. Materials and methods

2.1. Insects

Eggs of *H. armigera* were kindly provided by Prof. Jing-Lian Shen, Nanjing Agricultural University (Nanjing, China). Larvae were reared individually on an artificial diet at 25 ± 1 °C in 14 h light:10 h dark (a long day length) or at 20 ± 1 °C in 10 h light:14 h dark (a short day length). Under short day length, >95% of the pupae entered diapause, and diapause pupae were identified by examining the pupal eyespots (stemmata) and adult eclosion as described previously (Phillips and Newsom, 1966). Under long day conditions, all pupae were nondiapause.

The developmental stages were synchronized at each molt by collecting new larvae or pupae at daily intervals. The brain and other organs were dissected in 0.75% NaCl, and stored at −70 °C until use.

2.2. RNA isolation, cDNA synthesis and polymerase chain reaction (PCR)

Total RNA was isolated from the brain of *H. armigera* pupae by using an acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). The 20 brains were homogenized in Solution D (Chomczynski and Sacchi, 1987), placed on ice for 5 min and then sodium acetate and chloroform/isoamylalcohol (49:1) were added. The mixture of homogenized brains was centrifuged at $10{,}000 \times g$ at 4 °C for 20 min. The supernatant was transferred into a new tube, and then the isopropanol was added. After centrifugation, the RNA pellet was washed in 75% ethanol and then dissolved in the ddH_2O. One microgram of total RNA was reverse transcribed at 42 °C for 1 h in a 10 μl final volume reaction mixture containing reaction buffer, 10 mM DTT, 0.5 mM dNTP, 0.5 μg oligo-dT18, and reverse transcriptase from avian myeloblastosis virus (AMV, Takara, Japan).

Two degenerate primers DPTF (5′-ATAAT(G/C)CAGTCA(T/C)T(G/A)(G/A)TGCC-3′) and DPTR (5′-GTTTC(C/T)TTGCAAA(C/T)GTA(C/G)GG-3′) were designed based on the conserved amino acid sequences of known PTTHs (Kawakami et al., 1990; Sauman and Reppert, 1996; Sehnal et al., 2002; Shionoya et al., 2003). DPTF was derived from the conserved sequence motif Q/RSL/FM/VPKT/AM, while DPTR was derived from the sequence PYV/ICKES/T (Fig. 2). PCR reaction was performed with primers DPTF and DPTR under the following conditions: three cycles of 40 s at 94 °C, 40 s at 45 °C, 45 s at 72 °C, then 30 cycles of 40 s at 94 °C, 40 s at 47 °C, 45 s at 72 °C. A weak DNA band corresponding to approximately 480 bp of the expected size was excised from the agarose gel and purified using a DNA gel extraction kit (Takara, Japan).

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