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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.

Keywords: Prothoracicotropic hormone; cDNA structure; Developmental expression; *Helicoverpa armigera*

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).

2.3. Rapid amplification of cDNA ends (RACE)

For each 5′- and 3′-RACE, the cDNA was synthesized according to the manufacturer's protocol (SMART^{TM} kit, Clontech). Specific primers PTR1 and PTR2 for 5′-RACE, and PTF1 and PTF2 for 3′-RACE were synthesized based on the cDNA sequences obtained from PCR with degenerate primers. 5′-RACE amplification was performed on 2.5 μl of 5′-ready-cDNA with Universal Primer Mix (UPM, Clontech) and PTR1, then the nested PCR was carried out with Nested Universal Primer A (NUP, Clontech) and PTR2. 3′-RACE amplification was performed on 2.5 μl of 3′-ready-cDNA with UPM and PTF1, then with NUP and PTF2. The PCR conditions were as follows: after 5 min 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 60 s at 72 °C, then 10 min at 72 °C.

2.4. Sequence analysis and phylogenetic tree construction

PCR products were separated on an agarose gel, and a single band was isolated by DNA gel extraction kit (Takara, Japan). The purified PCR product was ligated into T-vector (Takara, Japan) and then transformed into XL-1 blue competent bacteria. The positive recombinant clone with an insert was sequenced using the dideoxynucleotide chain termination method (Takara, Dalian, China).

Based on the amino acid sequences of the PTTH precursor, a phylogenetic tree (neighbor-joining, NJ) was constructed using CLUSTALX program (Thompson et al., 1997).

2.5. Northern blot analysis

Total RNA (20 μg) was subjected to electrophoresis using a 1.2% agarose gel containing formaldehyde and transferred to a nylon membrane (Hybond N^+, Amersham). The nylon membrane was prehybridized for 6 h followed by addition of radiolabeled PTTH cDNA as a hybridization probe for 20 h at 45 °C in 5 × SSPE (saline sodium phosphate ethylenediaminetetraacetic acid; 1 × SSPE = 180 mM NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM EDTA) containing 50% formamide, 5 × Denhardt's solution, 0.1% SDS and 100 μg/ml denatured salmon sperm DNA (Sambrook et al., 1989). After hybridization, the membrane was washed with 0.2 × SSPE at 45 °C and finally exposed to an X-ray film for 24 h at −70 °C.

2.6. Developmental expression

The developmental expression of PTTH mRNA was measured with the combined methods of quantitative RT-PCR and Southern blots according to previously described procedures (Xu et al., 2003). Total RNA was extracted from 20 brains, and 1 ng of rabbit globin (RG) mRNA/20 brains was added as an internal standard (Xu et al., 1995). The first strand cDNA was synthesized from 1 μg of total RNA at 42 °C for 1 h, prepared with an AMV reverse transcript system kit (Takaka, Japan). The PTTH cDNA fragment was amplified with the primers PP1 and PTR1 (Fig. 1) for 20 cycles to assure that the reaction was in the linear range, based on our preliminary experiment (data not shown). The PCR products were electrophoresed on a 1.2% agarose gel and transferred to a Hybond-N^+ membrane. The 480 bp fragment corresponding to nucleotide position 21–500 of the PTTH cDNA was labeled with [α-^{32}P]-dCTP using a random primed DNA labeling kit (Takara, Japan). Southern hybridization with the labeled PTTH cDNA as a probe and signal detection were the same as described for the Northern blots.

2.7. Production of polyclonal antisera and Western blot analysis

Two types of polyclonal antisera recognizing PTTH were raised against the synthetic peptide and recombinant PTTH (re-PTTH), respectively. The synthetic 20-residue peptide, which corresponded to the N-terminal 1–20 amino acid sequence of Hearm-PTTH, was coupled to bovine serum albumin (BSA, Sigma) by glutaraldehyde, and the conjugated peptide in Freund's complete adjuvant was injected into rabbits at multiple sites. The other procedures were according to the method reported by Sun et al. (2003). In addition, the full-length recombinant PTTH expressed in *E. coli* was used as an antigen to produce a polyclonal antibody.

Proteins for use in Western blotting were extracted from tissues by homogenization in phosphate-buffered saline (PBS) followed by centrifugation at 12,000 g for 20 min at 4 °C. The supernatants were dried and stored at −70 °C. Protein extracts mixed with SDS-PAGE sample buffer were boiled for 10 min, and immediately loaded on the 12% SDS-PAGE gel. After SDS-PAGE, the proteins were blotted on a PVDF membrane (Hybond-P, Amersham), and the membrane was then incubated with rabbit anti-synthetic PTTH antiserum ($\frac{1}{400}$) for 2 h at 37 °C. After washing in PBST (PBS-Tween), the membrane was incubated in secondary antibodies (HRP-conjugated goat anti-rabbit IgG, dilution $\frac{1}{2000}$) for 2 h at 37 °C, and then washed thoroughly in PBST. The binding was detected using a DAB stock stain kit (Sino-American Biotechnology Co., China).

2.8. Whole-mount immunocytochemistry

The distribution of PTTH immunoreactivity in the *H. armigera* brain was investigated using whole-mount immunocytochemistry according to the procedures

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the PTTH cDNA in *H. armigera*. The suggested start codon ATG and stop codon TAG are indicated in boxes. A putative polyadenylation signal is shown in bold letters. The endoproteolytic cleavage sites are shown by dotted lines. The predicted mature PTTH amino acid sequence is shaded. Arrows under the nucleotide sequences represent the position of the different synthetic primers used in PCR. Degenerate primers are 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′). Specific primers are PP1(5′-GGAGAAAGCTGTACAGTC-3′), PTF1(5′-AGTCAGTCGAAACGAGGA-3′), PTF2 (5′-AAGCATTACCGGATCCCT-3′), PTR1(5′-CTATGTAACGTGGGTAGGT-3′) and PTR2 (5′-ATCCGTTCTGTTTGGGCT-3′).

described in Westbrook et al. (1990). Briefly, the brain was fixed for 4 h in 4% paraformaldehyde in phosphate buffer (pH 7.4) and then soaked in PBS containing 2% TritonX-100 (PBST) for 4 h. Tissues were incubated in the primary antibodies (1:4000, anti-synthetic PTTH or 1:6000, anti-re-PTTH), and the secondary antibodies (1:2000) for 6 h, respectively. Tissues were twice washed in PBS-T and visualized with a DAB stock stain kit. The stained tissues were observed under a microscope equipped with a digital camera (Olympus BX60). The controls were performed with the same procedures, except the primary antibodies were replaced by the preimmunized rabbit serum.

2.9. Expression of recombinant PTTH in E. coli

To construct pET28A-PTTH, the mature PTTH-coding sequence was amplified by PCR using primers MPT5 (5′-CCGGAATTCGGTAACATTAAAGTC-GAG-3′) and MPT3 (5′-CCCAAGCTTGATAG-CATGTACAGGGCGTGTA-3′). The PCR product was inserted into pET28A with *Eco*R I and *Hin*d III sites, and transformed into *E. coli*, BL21 strain. After 3 h preincubation, the recombinant PTTH (re-PTTH) was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to give a final concentration of 0.5 mM for 4 h. The cells (1L) were harvested by centrifugation and re-PTTH was isolated from the inclusion body of the cells by sonication. After centrifugation, the supernatant was retained and loaded onto a nickel-nitriloacetic acid (Ni–NTA) column (2 cm × 3 cm, Qiagen). The column was washed with 50 ml of binding buffer (10 mM Tris, pH 7.5, containing 300 mM NaCl and 10 mM imidazole) followed by 200 ml of washing buffer (10 mM Tris, pH 7.5, containing 300 mM NaCl and 20 mM imidazole). The bound proteins were eluted with 60 ml of elution buffer (10 mM Tris, pH 7.5, containing 300 mM NaCl and 250 mM imidazole). The purified re-PTTH was dialyzed against 10 mM PBS for 36 h at 4 °C and then used for production of the polyclonal antibody and for the bioassays.

2.10. PTTH bioassay in vivo

To test the biological activity of purified re-PTTH, *H. armigera* diapausing pupae were injected with 10 ng of re-PTTH in 5 μl volume (diluted with distilled water). As a control, 5 μl distilled water was injected into diapausing pupae. For diapause termination experiments, the newly formed diapause-destined pupae were placed at low temperature (20 ± 1 °C) for 1 wk to allow the pupae to enter diapause and then injected with the re-PTTH and held at 25 ± 1 °C to evaluate the efficacy of the neuropeptide in terminating diapause.

3. Results

3.1. Cloning and characterization of Heamr-PTTH cDNA

Based on the sequences conserved among the PTTHs reported in other species, two degenerate primers DPTF and DPTR were designed and used for PCR amplification. A 473 bp cDNA fragment was amplified and cloned (Fig. 1). Sequence analysis revealed that the fragment was similar to the known PTTHs. Two pairs of specific primers (PTF1–PTR1, PTF2–PTR2) (Fig. 1) were then designed for 3′- and 5′-RACE based on the 473 bp cDNA sequences. After PCR amplification, a 481 bp fragment from 5′-RACE and a 458 bp fragment from 3′-RACE were obtained, with a 56 bp overlapping sequence (Fig. 1). The nucleotide sequence reported here is available in GenBank under accession number AY286543.

The full-length PTTH cDNA consists of 876 nucleotides and predicts an open reading frame (ORF) of 226 amino acids (Fig. 1). The ORF is terminated by a TAG stop codon that is followed by a 126 nucleotide 3′ untranslated region exclusive of the poly (A) tail. A consensus polyadenylation signal (AATAAA) is found at 99 bp from the stop codon. Hearm-PTTH preprohormone shares several conserved features, such as putative proteolytic cleavage sites (R^{45}–K^{46}–R^{47}, R^{113}–K^{114}–R^{115}), an N-glycosylation site (N^{157}–C^{158}–S^{159}) and seven cysteine residues. Mature Hearm-PTTH (111 amino acids) is derived by proteolytic processing of the preprohormone at R^{113}–K^{114}–R^{115} (Fig. 1).

In comparison with other known PTTHs, the Hearm-PTTH ORF is highly homologous to two other noctuid moths, *H. zea* (98%) and *H. virescens* (94%), but only 48–57% with bombycoid moths. The mature PTTH molecular identity is 99% for *H. zea*, 93% for *H. virescens*, 60% for *B. mori*, 54% for *M. sexta*, 50% for *A. pernyi*, 49% for *S. c. ricini* and *H. cecropia*, respectively (Fig. 2). Based on phylogenetic inference, Hearm-PTTH, along with the PTTHs from *H. viresces* and *H. zea*, form a cluster that is distinct from the other known PTTHs (Fig. 3).

3.2. Tissue expression of Hearm-PTTH mRNA

When total RNA (20 μg) from various tissues was subjected to Northern blot analysis using ^{32}P-labeled PTTH cDNA as a probe, the PTTH mRNA was detected only in the brain (Fig. 4). The size of the hybridized band was approximately 0.9 kb, a size consistent with the cloned Hearm-PTTH cDNA (876 bp). Thus, we conclude that the cloned cDNA represents the full-length mRNA of Hearm-PTTH preprohormone. We also examined the tissue specificity of PTTH mRNA using RT-PCR, a technique that is more sensitive than Northern blotting. A positive signal was present not only in the brain, but also in subesophageal ganglion (SG), thoracic ganglion (TG), abdominal ganglion (AG), midgut and fat body, although these bands were much weaker than in the brain. The PTTH mRNA was not detected in ovary, muscle or epidermis (Fig. 5).

3.3. Developmental changes of Hearm-PTTH mRNA

The developmental changes of PTTH mRNA in diapause and nondiapause individuals were measured using RT-PCR combined with Southern blots. In nondiapause-destined individuals, the PTTH mRNA content was consistently high from day 2 of the 6th instar to adult eclosion, with the exception of relatively low levels at the later instar larval stage and the onset of the wandering phase. By contrast, the PTTH gene expression in diapause-destined individuals was markedly lower at the onset of the wandering larval stage and remained low as the pupae entered diapause (Fig. 6A).

Diapause in this species can be broken by holding the pupae at 25 ± 1 °C for 8 wk. We investigated the changes of PTTH mRNA in pupae as they terminated diapause and initiated adult development, as indicated by migration and the eventual disappearance of the eyespots in the post-genal region. At the initiation of eyespot migration, the PTTH mRNA began to increase and reached a high level almost equal to that of day 2 nondiapause pupae by the time the eyespots disappeared (Fig. 6B). These results thus indicate that PTTH is indeed elevated again when diapause is terminated.

3.4. Distribution and localization of PTTH protein

Using an anti-PTTH antibody, Western blot analysis was performed to examine protein extracts from various neural tissues of *H. armigera* pupae. Under strong reducing conditions (10% β-mercaptoethanol), two weak bands were detected at 30 and 15 kDa in the brain (Fig. 7A), but no signal was observed from other

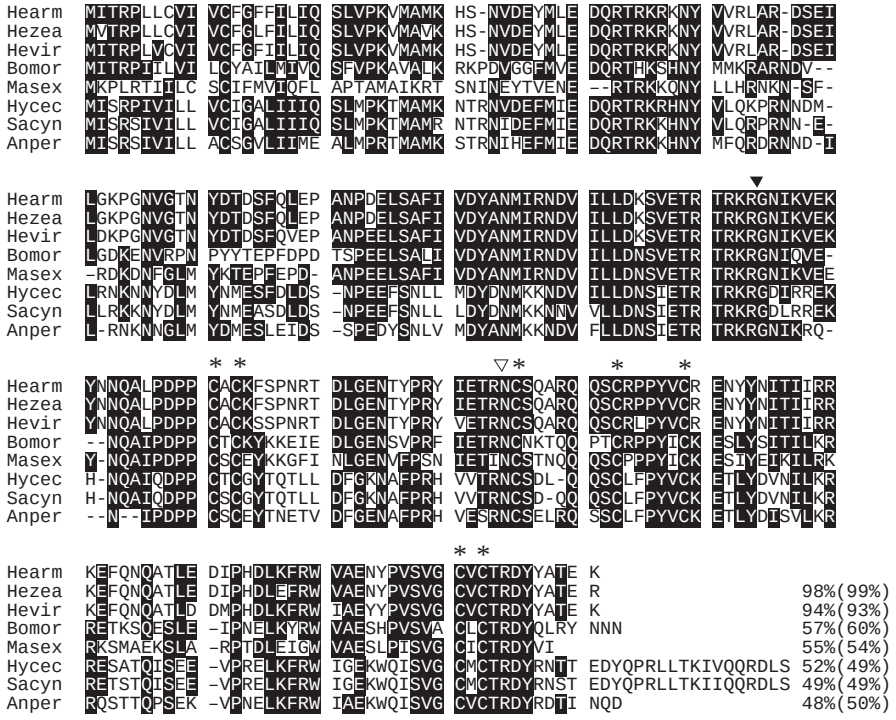

Fig. 2. Alignment of deduced amino acid sequences of eight PTTHs. Hearm, *H. armigera* (AY286543); Hezea, *H. zea* (AY172670); Hevir, *H. virescens* (AY172671); Masex, *M. sexta* (AY007724); Bomor, *B. mori* (D90082); Sacyn, *S. c. ricini* (L25668); Anper, *A. pernyi* (U62535); Hycec, *H. cecropia* (AF288695). Gaps are introduced in the sequences to maximize alignment. Highly conserved residues (more than 50% identity) are shaded. The filled triangles (▼) indicate the expected proteolytic processing sites for producing the mature PTTH. The conserved cysteine residues in the mature PTTH are indicated with an asterisk and the potential N-glycosylation site is indicated by the open triangle (▽). The percentages at the right indicate the amino acid identities of the preprohormone and mature PTTH (in brackets) compared to *H. armigera*, respectively.

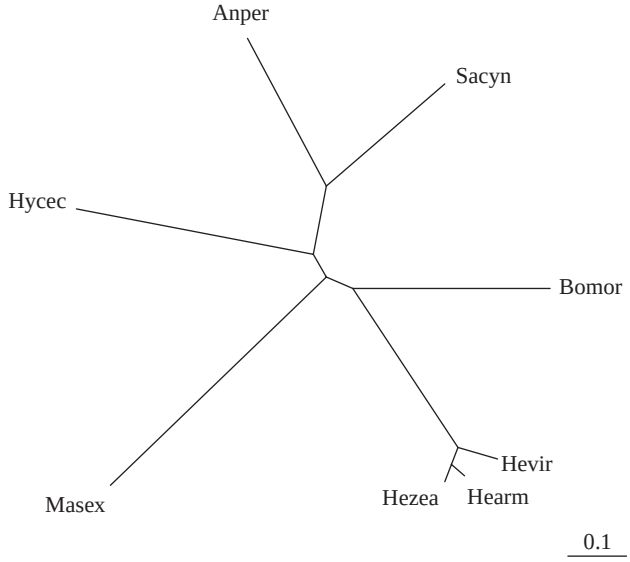

Fig. 3. Phylogenetic analysis by neighbor-joining (NJ) tree. The analysis is based on the amino acid sequences of the open reading frames of PTTH. Branch lengths are proportional to the number of amino acid substitutions.

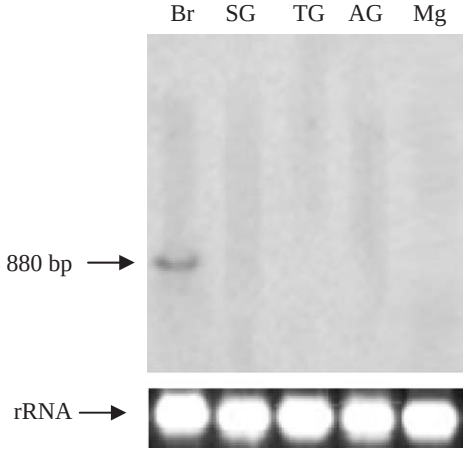

Fig. 4. Northern blot analysis. Total RNA (20 μg) was loaded on each lane and observed under UV light to verify equal loading. [α-^{32}P]-dCTP labeled Hearm-PTTH cDNA (corresponding to cDNA 21-500) was used as a probe. Br, brain; SG, subesophageal ganglion; TG, thoracic ganglion; AG, abdomen ganglion; Mg, midgut. The amount of ribosomal RNA (rRNA) loaded per lane (ethidium staining) served as a control for loading variation.

neural tissues, including SG, TG, and AG, which is consistent with the Northern blot analysis. When protein electrophoresis was performed under stronger conditions (15% β-mercaptoethanol), the 30 kDa almost disappeared (Fig. 7B). Thus, the 30 kDa band is likely to be the homodimeric PTTH as reported in *B. mori*

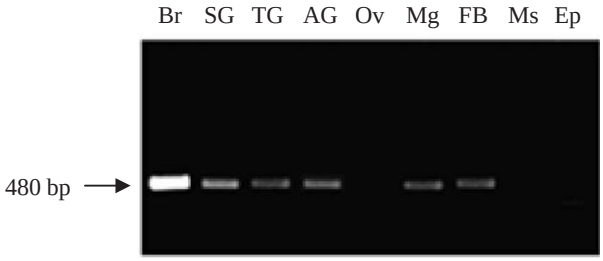

Fig. 5. Tissue distribution of Hearm-PTTH mRNA based on RT-PCR analysis. Total RNA extracted from the tissues was reverse transcribed and then subjected to 30 PCR cycles using PP1 and PTR1. Brain (Br), subesophageal ganglion (SG), thoracic ganglion (TG), abdomen ganglion (AG), midgut (Mg), fat body (FB) and epidermis (Ep) were dissected from larvae on day 3 of the 6th instar, and ovary (Ov) and muscle (Ms) were dissected from day 8 pupae and adults, respectively.

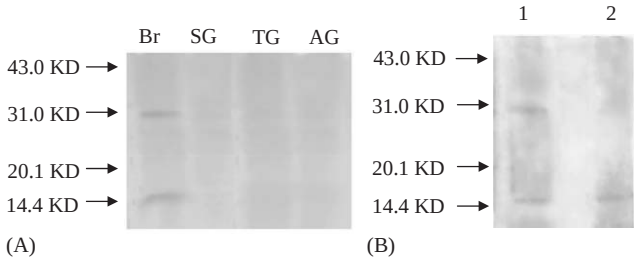

Fig. 7. Western blot analysis of Hearm-PTTH protein. (A) Tissue distribution of Hearm-PTTH. 20 μg of protein samples extracted from several different pupal tissues were loaded on each lane for immunoblot analysis. Br, brain; SG, subesophageal ganglion; TG, thoracic ganglion; AG, abdominal ganglion. (B) Western blot analysis under two different reducing conditions. 20 μg of protein sample was extracted from pupal brains. Lane 1, 5% β-mercaptoethanol; lane 2, 15% β-mercaptoethanol.

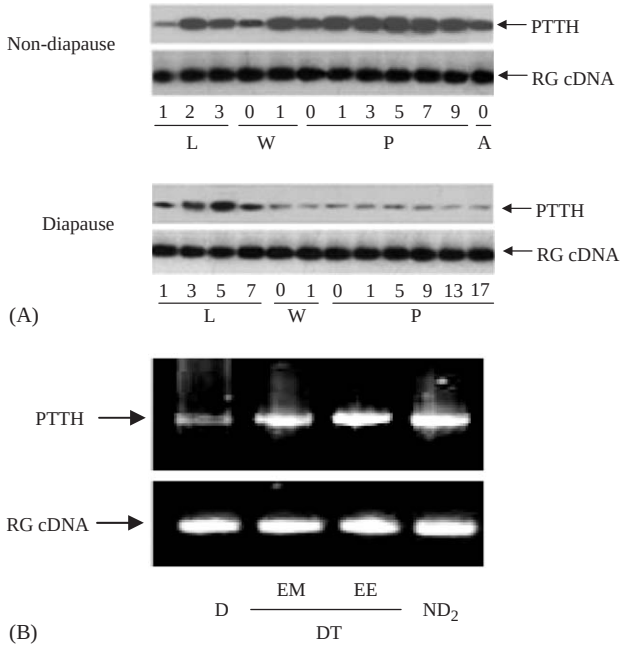

Fig. 6. (A) Developmental changes of PTTH mRNA. RNA was extracted from 20 brains or brain-SG complexes of larvae, wandering larvae, pupae and adults. One microgram of total RNA was subjected to reverse transcription and PCR amplification (20 cycles), then PCR products were electrophoresed on a 1.2% agarose gel and hybridized using labeled oligonucleotide as a probe. L, W, P and A represent larvae, wandering larvae, pupae and adults, respectively. The Arabic numerals indicate the day of larval, wandering larval, pupal and adult stages. RG, rabbit globin. (B) Changes of PTTH mRNA at diapause termination. RNA was extracted from 10 pupal brains and subjected to RT-PCR (30 cycles). The PCR products were electrophoresed on a 1.2% agarose gel. Diapause termination was checked by observing eyespot movement in the post-genal region of the pupae. D, diapausing pupae; DT, diapause termination pupae; ND_2, day 2 nondiapause pupae. EM and EE represent progressive stages of pharate adult development in which the eyespots have moved to the edge of the post-genal region or are no longer visible, respectively.

(Kawakami et al., 1990) and *M. sexta* (Shionoya et al., 2003).

PTTH neurosecretory cells were detected by whole-mount immunocytochemistry using two kinds of antisera, one raised against a synthetic PTTH fragment and the other against a re-PTTH. Both antibodies immunostained two pairs of dorsolateral neurosecretory cells in the *H. armigera* brain (Fig. 8). However, the quality of the antibody raised against the re-PTTH (Fig. 8B) was better than that raised against the synthetic PTTH fragment (Fig. 8A): both the PTTH neurosecretory cells and axonal projections could be clearly seen with the antibody raised against re-PTTH (Fig. 8B). In control experiments, the primary antibodies were replaced by preimmunized rabbit serum, and no immunohistochemical staining was seen. The corpora allata have previously been shown to be the neurohemal organ for PTTH release in *M. sexta* (Westbrook and Bollenbacher, 1990), *B. mori* (Ishizaki and Suzuki, 1994) and *S. c. ricini* (Yagi et al., 1995). In *H. armigera*, the immunoreactive material was also detected in the axons of these neurosecretory cells which run across the brain midline to reach the contralateral corpora allata (Fig. 8B), suggesting that, in this species also, PTTH is liberated into the hemolymph from the corpora allata.

3.5. Prothoracicotropic activity of re-PTTH

To obtain Hearm-PTTH protein for evaluation of prothoracicotropic activity, the predicted mature Hearm-PTTH was inserted into the plasmid vector pET28A and then transformed into *E. coli*, BL21 strain. The cells were frozen, thawed and disrupted by sonication. After centrifugation, the cell extractions from the supernatant and inclusion body were electrophoresed on SDS-PAGE. Both supernatant and inclusion body showed the expected band (15 kDa), but the band was stronger in the inclusion body than in the

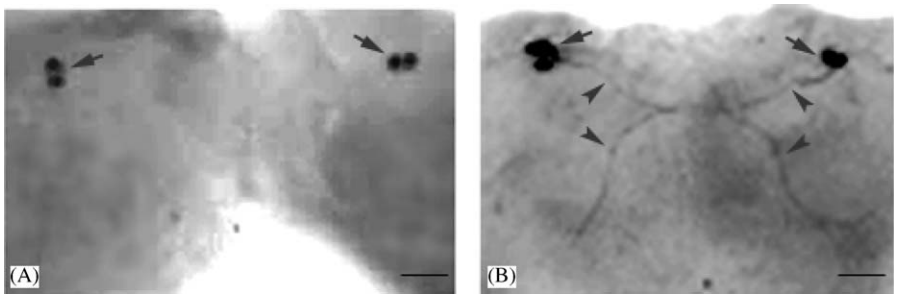

Fig. 8. Localization of PTTH immunoreactivity in the brain. The brain was dissected from day 2 nondiapause-destined pupae. Immunoreactivity detected with the (A) anti-synthetic PTTH polyclonal antibody and (B) the anti-re-PTTH polyclonal antibody. Positive cells (arrow) and axonal projections (arrowhead) are visible in the frontal view of the brain shown in B. Scale bars equal 30 μm.

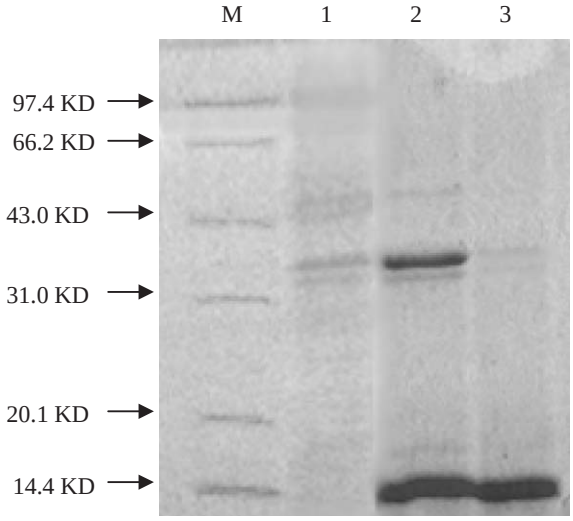

Fig. 9. Expression and purification of recombinant *H. armigera* PTTH. Protein extracts were mixed with SDS-PAGE sample buffer and loaded on an SDS-PAGE gel. Lane 1, the infected cell homogenate before being induced with IPTG; lane 2, the cell homogenate after being induced with IPTG; lane 3, re-PTTH purified with Ni–NTA column. M, protein markers.

supernatant. We then purified re-PTTH from the inclusion body using a Ni–NTA column (Fig. 9). To confirm that the expressed product was PTTH, Western blot was performed using the anti-PTTH antibody raised against synthetic PTTH. The result revealed a major PTTH-immunoreactive band with an apparent molecular weight of 15 kDa, which corresponds to the expected molecular weight. Control cells were transformed with the pET28A plasmid lacking the PTTH insert, but their lysates failed to generate a signal on Western blots using the anti-PTTH antibody (data not shown).

The purified re-PTTH was dialyzed and then assayed for biological activity. Diapausing pupae were injected with 10 ng re-PTTH/pupa or distilled water. Diapause could be terminated with an injection of re-PTTH (Table 1), thus clearly indicating that the cloned *H. armigera* PTTH cDNA encodes a functional hormone.

Table 1
Effect of recombinant PTTH on terminating pupal diapause in *H. armigera*

Treatment	Weeks after injection	
	2	3
Recombinant PTTH (10 ng in 5 μl)	14/20	14/20
Distilled water (5 μl)	0/20	4/20

The diapausing pupae were kept at low temperature (20 ± 1 °C) for 1 week after pupation, injected with the 5 μl solution, and then held at 25 ± 1 °C. The incidence of diapause termination is presented as the number of pupae terminating diapause/total number of injected pupae.

4. Discussion

In the present study, we obtained a cDNA that encodes a 876 bp PTTH preprohormone in the brain of *H. armigera*. Hybridization of pupal brain total RNA with a ^{32}P-labeled cDNA probe revealed a single distinct band. The hybridized transcript was approximately 0.9 kb, a size similar to the cloned *H. armigera* PTTH cDNA, thus indicating that the cloned cDNA represents the full-length PTTH mRNA. From the alignment of deduced amino acid sequences, the preprohomone and mature Hearm-PTTH show high homology with two other noctuid moths, *H. zea* and *H. virescens* (preprohomone PTTH, 98% and 94%; mature PTTH, 99% and 93% respectively), but low homology with species of Bombycoidea, *B. mori*, *S. c. ricini*, *A. pernyi*, *H. cecropia* and *M. sexta* (preprohomone PTTH, 48–57%; mature PTTH, 49–60%). Although the PTTH molecules show low identity between noctuid and bombycoid species, the PTTHs from all eight species share conserved structural features: proteolytic cleavage site for releasing mature PTTH, N-glycosylation site and seven cysteine residues presumably involved in establishing disulfide bonds between and within chains (Kawakami et al., 1990; Ishibashi et al., 1994). After cleavage at the site of R^{113}–K^{114}–R^{115}, the mature PTTH of *H. armigera* is released, with a monomeric

form of approximately 15 kDa, which has been confirmed by a Western blot. According to previous studies, the monomeric subunit refolds and a homodimer forms with the disulfide bonds, a feature that is essential for full prothoracicotropic activity (Ishibashi et al., 1994).

Northern blot analysis documented that Hearm-PTTH mRNA is expressed only in the brain as previously reported in Bomor-PTTH (Adachi-Yamada et al., 1994), Anper-PTTH (Sauman and Reppert, 1996) , Hezea-PTTH (Xu et al., 2003) and Hevir-PTTH (Xu and Denlinger, 2003). Western blot analysis showed the brain to be the expression site for the PTTH protein. By whole-mount immunocytochemistry, the Hearm-PTTH protein could be detected in two pairs of lateral protocerebral neurosecretory cells, as previously noted in *B. mori* (Aizono et al., 1997) and *M. sexta* (O'Brien et al., 1988; Westbrook et al.,1990). However, low levels of the Hearm-PTTH mRNA were also detected in additional tissues by PCR, as reported in *B. mori* (Adachi-Yamada et al., 1994) and *H. virescens* (Xu and Denlinger, 2003). Adachi-Yamada et al. (1994) indicated that the brain is the major site for Bomor-PTTH gene expression, but low levels of expression were also noted in the gut, epidermis and silk gland. In addition to strong PTTH mRNA expression in the brain of *H. virescens*, low levels of expression were detected in the SG, midgut and malpighian tubules (Xu and Denlinger, 2003). The function of PTTH produced in these other organs still remains unclear.

Previous studies have demonstrated that PTTH regulates insect development by stimulating PGs to synthesize and secrete ecdysteroids. In pupal diapause, PTTH is thought not to be released, resulting in a failure of the PGs to synthesize the ecdysteroids needed to initiate adult development (Denlinger, 1985, 2002). Expression analyses during pupal development showed that PTTH mRNA was much more weakly expressed in diapause-destined pupae and those that have entered pupal diapause, whereas the PTTH mRNA was highly expressed in pupae not destined for pupal diapause. This low level of expression during pupal diapause is similar to that observed in *H. virescens* (Xu and Denlinger, 2003). We now also provide critical evidence showing that PTTH mRNA increases again when diapause is terminated. Thus the data from both *H. virescens* and *H. armigera* support the idea that a failure of PTTH production and release is an essential component of the diapause regulatory mechanism. In the present study, this concept is further supported by the fact that an injection of re-PTTH will break the diapause of *H. armigera*. This view is also consistent with observations that PTTH can stimulate adult development in de-brained *A. pernyi* pupae (Sauman and Reppert, 1996) and stimulate ecdysteroidogenesis in the prothoracic glands of *B. mori* (Kawakami et al., 1990) and *M. sexa* (Gilbert et al., 2000).

The puzzling component is that DH can also break pupal diapause in *Helicoverpa*/*Heliothis* moths (Xu and Denlinger, 2003; Zhang et al., 2004a; Zhao et al., 2004), and Hearm-DH can stimulate PGs to synthesize and secrete ecdysteroids (Zhang et al. 2004b). What remains unclear is how these two neuropeptides can elicit the same response. Either neuropeptide by itself can fully terminate diapause. Does this represent two alternate mechanisms to achieve the same end? Earlier experiments by Meola and Adkisson (1977) demonstrated that *H. zea* can terminate diapause in the absence of the source of PTTH, the brain. The capacity of DH from the SG to terminate diapause could explain that observation, but in unoperated pupae, both neuropeptides are presumably available. Perhaps the most likely scenario is that both of these hormones normally act together to finely regulate the diapause in these noctuid pupae. Our current study provides critical molecular tools that will hopefully enable us to eventually resolve this intriguing conundrum.

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