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Functional analysis of the SGNP I in the pupal diapause of the oriental tobacco budworm, *Helicoverpa assulta* (Lepidoptera: Noctuidae)

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Abstract

Helicoverpa assulta suboesophageal ganglion neuropeptide I (Has-SGNP I) is a 24-amino acids peptide amide, which shows 62.5% similarity with the diapause hormone of *Bombyx mori* (Bom-DH). It has been demonstrated that embryonic diapause is induced by DH in *B. mori*. Injection of synthetic amidated Has-SGNP I terminated pupal diapause in a dose-dependent manner. Therefore, Has-SGNP I might be referred to a "diapause termination hormone" in *H. assulta* (Has-DTH). The maximal dose of Has-DTH for diapause termination was 1.0 μg and the half-maximal dose 0.4 μg. The time required for diapause termination of Has-DTH was 2–3 days longer than that of 20-hydroxyecdysone. During the pupal stage, DTH mRNA content in the SGs of nondiapausing pupae was always higher than in diapausing pupae using the combined method of quantitative RT-PCR and Southern blot. DTH gene also expressed at a low level while diapausing pupae were chilled at 4 °C, but increased rapidly and largely after being transferred to 25 °C. Using a competitive ELISA, Has-DTH-like immunoreactivity in the haemolymph showed the same pattern as that of Has-DTH gene expression. Those results indicated that Has-DTH gene expression was related to diapause development and could be activated by low temperature. Has-DTH might be useful to elucidate the mechanism of diapause termination in pupal diapause species.

Keywords: Suboesophageal ganglion neuropeptide I; Pupal diapause; Diapause termination hormone; *Helicoverpa assulta*

1. Introduction

Insect neuropeptides regulate various aspects of homeostasis and development functions as neurohormones, neuromodulators or neurotransmitters [1,2]. In lepidopteran insects, the suboesophageal ganglion (SG) produces several distinct neurohormones in response to specific developmental stages or to changes of environmental conditions [3–5].

Among these neurohormones, diapause hormone (DH) and pheromone biosynthesis activating neuropeptide (PBAN) are very important and well studied in several Lepidopteran species. DH from the silkworm, *Bombyx mori* (Bom-DH), induces embryonic diapause by acting on ovaries during pupal–adult development [6]. PBAN controls sex pheromone production by the pheromone gland in the female adults of Lepidoptera, such as the corn earworm, *Helicoverpa zea* [7], the silkworm, *B. mori* [8,9], the gypsy moth, *Lymantria dispar* [10], the tobacco budworm, *Helicoverpa assulta* [11], the black cutworm moth, *Agrotis ipsilon* [12], the Egyptian armyworm, *Spodoptera littoralis* [13], the cotton bollworm, *Helicoverpa armigera* (GenBank accession no. AY043222), and the tobacco hornworm, *Manduca sexta* (GenBank accession no. AY172672).

A number of studies have shown that DH and PBAN both require the C-terminal FXPRLamide as an essential core structure for expression of biological activity [14–17]. In *B. mori*, molecular cloning reveals that a single gene encodes a common polyprotein precursor, from which DH, PBAN and three other members of FXPRLamide family are produced [18]. Therefore, this cDNA was designed as DH-PBAN cDNA [19]. Similarly, PBAN cDNAs from *H. zea* and *H. assulta* also encode a polyprotein precursor containing a DH-like peptide. However,

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Has SGNP **I**	N D V K D G A A S G A H S D R L G L W **F G P R L**	100%
Har DTH	N D V K D G A A S G A H S D R L G L W **F G P R L**	100%
Hez PGN-23	N D V K D G A A S G A H S D R L G L W **F G P R L**	100%
Bom DH	T D M K D E S D R G A H S E R G A L W **F G P R L**	62.5%
Consensus	- D - K D - - - - G A H S−R - - L W **F G P R L**	

Fig. 1. Comparison of Has-SGNP I sequence to other known DH or DH-like peptide. Has, *H. assulta*; Har, *H. armigera*; Hez, *H. zea*; Bom, *B. mori*. SGNP, suboesophageal ganglion neuropeptide; DTH, diapause termination hormone; PGN, PBAN-encoding gene neuropeptide; DH, diapause hormone. The FXPRL C-terminus of the peptides were showed by bold letters. The percentage represents the homology with Has-SGNP I.

these authors still called them PBAN cDNAs [5,11], probably because the physiological functions of the DH-like peptides are unclear in these species. In *H. assulta*, the DH-like peptide encoded by the PBAN cDNA was named Has-SGNP I [11] and its potential physiological function has never been studied.

Recently, a cDNA homologous to Bom-DH-PBAN, Hez-PBAN and Has-PBAN cDNAs was isolated from *H. armigera* (GenBank accession no. AY043222), a close relative of *H. assulta* [11]. It encodes a polyprotein precursor for five FXPRLamide peptides. One of them, with 24 amino acids (N^{24}–L^{47}), is identical to Has-SGNP I and similar to Bom-DH in amino acid sequence (Fig. 1). This neuropeptide has biological activity of diapause termination in *H. armigera* (Har-DH) [20]. Thus, we suppose that Has-SGNP I might also have a similar function of diapause termination in *H. assulta*.

To test this hypothesis, in the present study, we report the functional analysis of Has-SGNP I on diapause termination of *H. assulta*. Developmental changes of Has-SGNP I mRNA in the SGs were investigated by the combined method of quantitative RT-PCR and Southern blot, and developmental changes of Has-SGNP I immunoreactivity in the haemolymph were also measured by competitive ELISA. All these data suggested that Has-SGNP I was also involved in terminating the pupal diapause in *H. assulta* as Har-DH in *H. armigera*. Therefore, Has-SGNP I might be referred to a "diapause termination hormone" in *H. assulta* (Has-DTH).

2. Materials and methods

2.1. Animals

Larvae of *H. assulta* were collected from a pepper field at the suburb of Zhengzhou, Henan, China. Their offspring were reared on an artificial diet [21]. Diapausing pupae (DP) and nondiapausing pupae (NDP) were obtained by rearing larvae at 22 °C under 10 h light–14 h dark (10L–14D) and at 25 °C under 14 h light–10 h dark (14L–10D) photoperiods, respectively. Diapausing pupae were checked by the retention of eyespot (stemmata) movements in the post-genal region of the pupae [22]. SGs were dissected out and the haemolymph was collected from the diapausing and nondiapausing pupae, and stored at −70 °C until used.

2.2. Injection of Has-SGNP I and 20-hydroxyecdysone

Has-SGNP I with amidated C-terminus (Has-SGNP I-NH_2) and free acid C-terminus (Has-SGNP I-COOH), which are deduced from Has-PBAN cDNA [11], were synthesized at the Genemed Synthesis (USA). Has-SGNP I and 20-hydroxyecdysone (Sigma, St. Louis, MO) were dissolved in distilled water and stored at −20 °C until used. An aliquot (5 μl) of solution containing different amounts of Has-SGNP I or 20-hydroxyecdysone was injected into each diapausing pupa with a fine glass capillary. New pupae were incubated at 22 °C (10L–14D) for 1 week, and then were placed at 25 °C for 5 days to check diapause status. These diapausing pupae were injected with Has-SGNP I and 20-hydroxyecdysone, respectively. Values were analyzed using Microsoft Origin 6.0.

2.3. RT-PCR amplification and quantification

Total RNA was extracted from the SGs of diapausing and nondiapausing pupae by the single-step method of acid guanidinium thiocyanate–phenol–chloroform extraction [23]. One nanogram of rabbit globin (RG) mRNA/15SGs was added to the extraction mixture as an internal standard [24].

The first strand cDNA was synthesized from 1 μg of total RNA at 42 °C for 1 h prepared with a 'TaKaRa AMV Reverse Transcript system Kit' (TaKaRa, Japan). PCR was performed with primers P_1 (5′-CTTGCTGTCTTCACTAC-GAG-3′, nt 31–50 of Has-PBAN cDNA) and P_2 (5′-TGAACTCCACGTTTTCGAAG-3′, nt 339–358 of Has-PBAN cDNA) for 18 cycles in a DNA Thermal Cycler 480 (Perkin-Elmer), which assured the reaction being 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 Hybond-N^+ membrane (Amersham). The membranes were probed with a segment of the Has-PBAN cDNA (nt 12–527) labeled with [α-^{32}P]dCTP using a random primer DNA labeling kit

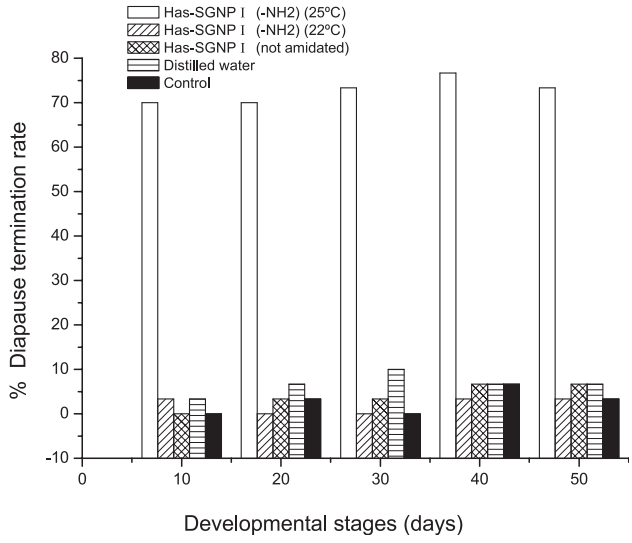

Fig. 2. Effect of Has-SGNP I injection on termination of pupal diapause. Has-SGNP I (200 pmol/pupa) was injected into the diapausing pupae at 22 or 25 °C at different pupal stages (days 10, 20, 30, 40, 50). The ratio of diapause termination is present by the number of diapause terminated pupae/total number of treated pupae. Control means that the pupae had no treatment. Each point represents the mean values with three repeats (10 pupae/each repeat) for each treatment.

(TaKaRa). The radioactive bands were quantitated with a phosphorimager (Typhoon 8600, Amersham-Pharmacia).

2.4. Competitive ELISA

The method of competitive ELISA was adopted from Ma et al. [25] with some modifications. The antiserum against Har-DH was a gift from Jiu-Song Sun, University of Science and Technology of China. A total of 50 μl of haemolymph was extracted from each pupa. A haemolymph mixture of 10 pupae was applied as a sample for the competitive ELISA. Three samples were tested at each point. All procedures were performed at room temperature. For the analysis of competitive ELISA, the coating plates were prepared with Has-SGNP I-NH_2 (5 pmol/well) in 0.05 M sodium carbonate–bicarbonate buffer (pH 9.3) overnight and blocked with 2% skimmed milk for 1 h. Plates were incubated with 30 μl of the diluted antiserum against Har-DH (1:3000 final concentration in $PBST_w$ with 2% skimmed milk) for 1.5 h. Simultaneously, standard peptides (0.002–1 pmol/well) and the haemolymph samples (1:20) were added respectively in a volume of 50 μl/well. Plates were washed three times with PBS-T_w after each incubation. Afterwards, plates were incubated with a secondary antibody (anti-rabbit goat IgG labeled with horseradish peroxidase, Promega) (1:20,000 final concentration in $PBST_w$ with 2% skimmed milk) for 2 h. The 100 μl of the substrate mixture (4 mg *o*-phenylenediamine dihydrochloride, 0.1% H_2O_2) was added and incubated for 15 min. The reaction was stopped with 50 μl of 2 M H_2SO_4. Finally, the results were measured at 490 nm using an automated microplate reader (ELx 800, Bio-Tek Instruments). Values which coincided with Has-SGNP I-NH_2 standard curve were analyzed using Microsoft Origin 6.0.

3. Results

3.1. Biological activity of Has-SGNP I on diapause termination

In this experiment, Has-SGNP I-NH_2 (200 pmol/pupa), Has-SGNP I-COOH (200 pmol/pupa) and distilled water were injected respectively into the diapausing pupae at different developmental stages (10–50 days after pupation), which were incubated at 22 °C for more than one week and then transferred to 25 °C for 5 days to check diapause status. For Has-SGNP I-NH_2, 22 °C was also used. Intact pupae were used as a control group. The results were shown in Fig. 2.

At 25 °C, after injection of Has-SGNP I-NH_2, 70–80% pupae broke diapause efficiently and developed to adults within thirteen days. No significant difference of diapause termination (0–10%) was observed among the treatments of Has-SGNP I-COOH and distilled water as well as the control of intact pupae. Those data suggested that Has-SGNP I-NH_2 might be called a "diapause termination hormone" (Has-DTH), and the amidated C-terminus of Has-SGNP I was an indispensable element to diapause termination activity. In addition, at 22 °C, Has-SGNP I-NH_2 had no diapause termination activity on the diapausing pupae.

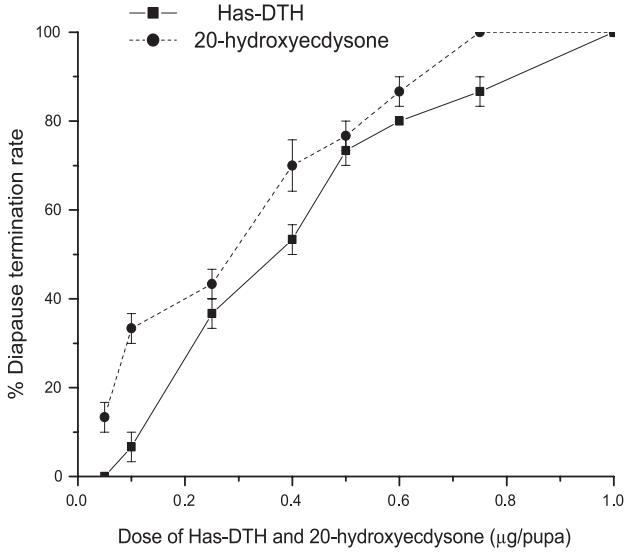

Fig. 3. Dose–response relationships of diapause termination to Has-DTH and 20-hydroxyecdysone. Has-DTH and 20-hydroxyecdysone were diluted and injected into diapausing pupae. Each point represents the mean values (± S.D.) by a vertical bar, with three repeats (10 pupae/each repeat) for each determination.

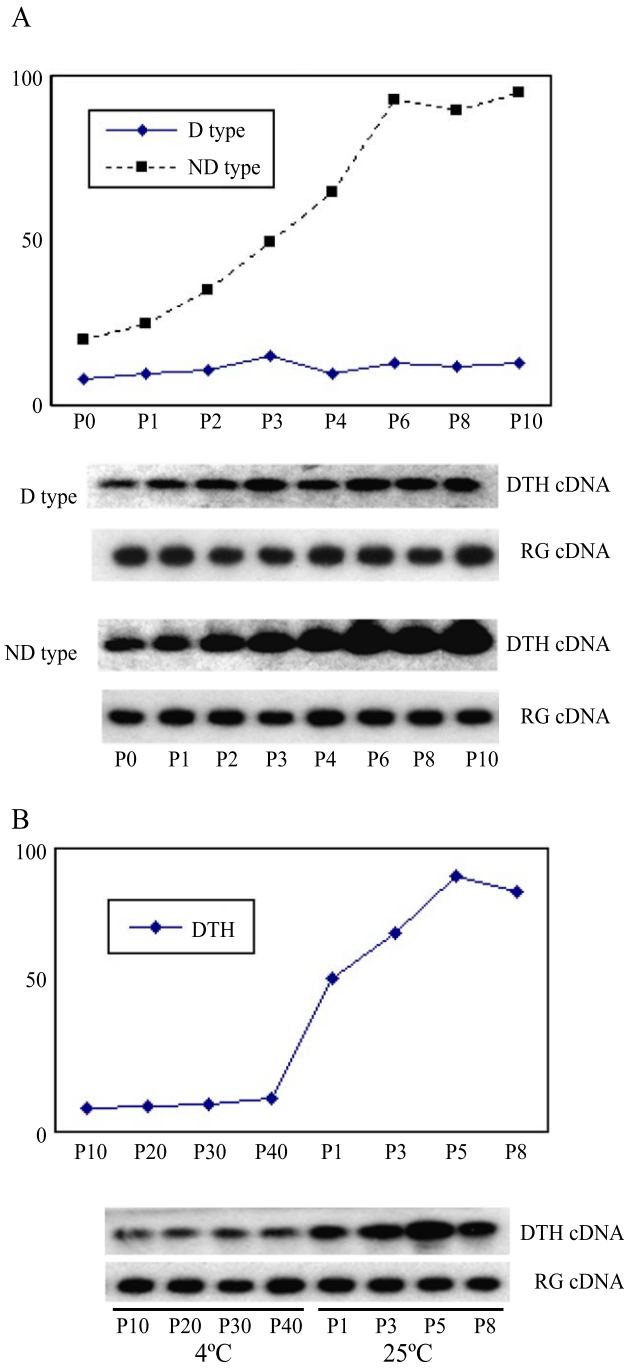

Fig. 4. (A) Changes of Has-DTH mRNA content in diapausing and nondiapausing pupae of *H. assulta*. P0–P10 represent days after pupation. (B) Changes in Has-DTH mRNA during the 4 °C chilled and later in 25 °C. The SGs were dissected from the diapausing pupae chilled at 4 °C and then moved to 25 °C. P10–P40 represent days chilled at 4 °C; P1–P8 represent days transferred to 25 °C. Total RNA extracted from SGs (15 pupae) was subjected to RT-PCR amplification (18 cycles). PCR products were electrophoresed and hybridized using radiolabeled Has-PBAN cDNA as a probe. 1ng of rabbit globin (RG) mRNA was used as an internal standard in each extraction. The radioactive bands were quantitated with a phosphorimager and corrected by radioactivity recovered in RG mRNA, which was added as an internal standard. The results are shown as the amounts of Has-DTH mRNA relative to the highest value (100%). The quantitative RT-PCR was repeated three times. D type and ND type represent diapause-destine and nondiapause-destine pupae, respectively.

3.2. Dose–response curves of Has-DTH and 20-hydroxyecdysone

The dose–response relationships were estimated by injecting different doses of Has-DTH and 20-hydroxyecdysone into diapausing pupae. The dose–response curves of Has-DTH and 20-hydroxyecdysone to terminate pupal diapause were shown in Fig. 3. For Has-DTH, all diapausing pupae were broken by injecting ≥ 1.0 μg/pupa (400 pmol/pupa). The half-maximal value was estimated to be 0.4 μg/pupa (150 pmol/pupa). Diapause was usually terminated on day 5 after the injection. On the other hand, for 20-hydroxyecdysone, the complete termination of diapausing pupae was reached by injecting ≥ 0.75 μg/pupa (300 pmol/pupa). The half-maximal value was estimated to be 0.3 μg/pupa (120 pmol/pupa). Diapause was usually terminated on day 2 or 3 after the injection. These data proved that Has-DTH had the same physiological function on diapause termination as 20-hydroxyecdysone.

3.3. Expression of Has-DTH gene

Developmental expression of Has-DTH gene during the pupal stage was examined using the combined method of quantitative RT-PCR and Southern blot [24]. DTH mRNA content in the SGs of diapause pupae remained low till day 10. During the early stages of nondiapausing pupae, DTH mRNA content increased gradually and reached a high level at the late stage of pupal–adult development. Comparatively, DTH mRNA content in the SGs of nondiapausing pupae

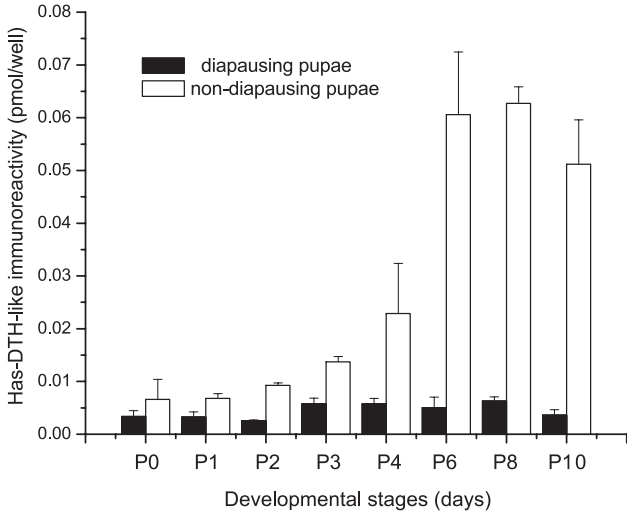

Fig. 5. Developmental changes of Has-DTH-like immunoreactivity in the haemolymph of the diapausing and nondiapausing pupae in *H. assulta*. The haemolymph samples were collected from the diapausing and non-diapausing pupae, and analyzed by ELISA as described in Section 2. Each well was added into 2.5 μl of the haemolymph sample. Results are expressed as amount per well by compared to the standard value. P0–P10 represent days after pupation. The haemolymph of 10 pupae were mixed as a sample for competitive ELISA and each point represents the mean values from three samples with the S.D. shown by a vertical bar.

was much higher than in diapausing pupae at the same developmental stage (Fig. 4A).

DTH mRNA content did not increase significantly when the diapausing pupae were chilled at 4 °C and diapause was being gradually broken. Once the diapausing pupae were transferred from 4 to 25 °C, DTH mRNA content rapidly increased (Fig. 4B). Again, this experiment indicated that DTH gene expression might closely relate to diapause termination and continuous development.

3.4. Developmental changes of Has-DTH in the haemolymph

Since Has-DTH has an identical sequence to Har-DH (Fig. 1), the antiserum against Har-DH was used as the primary antibody in the competitive ELISA. A plate coating Has-DTH of 5 pmol/well and the primary antiserum dilution of 1:3000 were optimal. These values were used in all of the ELISAs reported here. Has-DTH-like amounts in the haemolymph of diapausing and nondiapausing pupae were quantified by the competitive ELISA.

In the haemolymph of day 0 diapausing and nondiapausing pupae, DTH-like titer was measured to be 1.40 and 2.80 fmol/μl, respectively. During the late stages of pupal–adult development, for the haemolymph of day 8 diapausing pupae and nondiapausing pupae, DTH-like titer was estimated to be 2.59 and 26.32 fmol/μl, respectively (Fig. 5). Thus, from the early stage to late stage of pupal–adult development, DTH-like immunoreactivity was 2–10-fold higher in the haemolymph of nondiapausing pupae than that of diapausing pupae. Using the same procedure as described above, we also measured the DTH-like titer of haemolymph from diapausing pupae incubated at 4 °C and then transferred these pupae to 25 °C. DTH-like titer of the diapausing pupae on day 8 at 25 °C were three- to six-fold higher than on days 10 and 40 at 4 °C, respectively (Fig. 6). These results confirmed further that DTH-like titer in the haemolymph was also correlated with diapause termination.

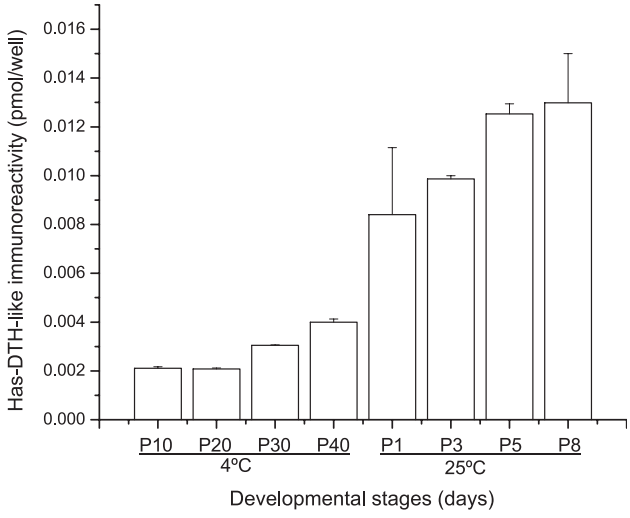

Fig. 6. Developmental changes of Has-DTH-like immunoreactivity in the haemolymph of the diapausing pupae chilled at 4 °C and then moved to 25 °C. P10–P40 represent days chilled at 4 °C; P1–P8 represent days transferred at 25 °C. The haemolymph samples collection and analysis are the same as described above.

4. Discussion

H. assulta belongs to insect species with pupal diapause [26], which is controlled by prothoracictropic hormone (PTTH) and molting hormone (MH or ecdysone). Pupal diapause is usually maintained by a decrease in the haemolymph ecdysteroid titer, due to a decrease in the biosynthetic activity of the prothoracic gland, which produces ecdysone. Such a decrease is caused by depletion of PTTH, a neuropeptide that is produced by two pairs of neurosecretory cells in the brain and stimulates the prothoracic glands to secrete ecdysone. Diapausing pupae can be stimulated to initiate development at any time by implantation of an "active" brain or by a simple injection of 20-hydroxyecdysone [3]. For some insect species, if pupae are exposed to a long period of cold, almost all individuals could break diapause within a few days after they are returned to warm temperatures [27].

Recent advances in insect neuropeptides have suggested that a group of neuropeptides possessing FXPRLamide at their C-termini are widely distributed among insects, such as DH, PBAN, myotropins and pyrokinins, which regulate diverse physiological processes [6,28,29]. DH and PBAN respectively control embryonic diapause in *B. mori* and pheromone biosynthesis in Lepidoptera moths. Myotropins and pyrokinins are involved in stimulating the hindgut of *Leucophaea maderae* and the oviduct of *Locusta migratoria* [29]. Meanwhile, myotropins and pyrokinins from the locust, *L. migratoria*, showed a weak activity of diapause induction in *B. mori* [30]. We previously reported that Has-SGNP I has the potential for inducing embryonic diapause in *B. mori*, but it could not induce pupal diapause in *H. assulta* [31]. The fact that DH-like peptide from *H. armigera* could break pupal diapause inspired us to examine the function of Has-SGNP I on diapause termination [20].

The present study has clearly indicated that Has-SGNP I could break pupal diapause and the C-terminal FXPRLamide was indispensable for the biological activity of diapause termination (Fig. 2). Noticeably, the diapause-terminating activity of Has-SGNP I was somehow lower in *H. assulta* than in *H. armigera*. In *H. armigera*, Har-DH (100 pmol/pupa) could completely terminate pupal diapause[20]. In any cases, Has-SGNP I definitely had a physiological function of diapause termination in *H. assulta*. Thus, we propose that Has-SGNP I in *H. assulta* might be called the diapause termination hormone (Has-DTH).

As reported here, Has-DTH had diapause terminating activity, but the duration, which could terminate diapause by injection of Has-DTH, was 2–3 days longer than by 20-hydroxyecdysone. We speculate whether DTH directly or

indirectly act on prothoracic glands to release ecdysone, and then stimulate pupae to break diapause. Meola and Gray [32] have demonstrated that haemolymph from nondiapausing pupae donors of *H. zea* stimulated ecdysone synthesis when injected into dipausing pupae. They concluded that a humoral factor was present in the haemolymph of nondiapausing pupae, and was required for diapause termination. In *M. sexta*, some factors in both the haemolymph and fat body stimulate the prothoracic glands to produce ecdysone in vitro [4]. Based on the previous results, we suggest that Has-DTH might be a haemolymph factor to terminate pupal diapause, after it was synthesized from the suboesophageal ganglion and released into the haemolymph in *H. assulta*. However, this hypothesis needs a lot more experiments to certify since it is still possible that Has-DTH might have a distinct mechanism from 20-hydroxyecdysone.

In the study presented here, Has-DTH mRNA content was much higher in SGs of the nondiapausing pupae than that of the diapausing pupae (Figs. 4A and 5). This offers a clue that the developmental regulation of DTH gene seems to depend on the physiological nature of *H. assulta*. In support of this hypothesis, Has-DTH-like immunoreactivity in the haemolymph was demonstrated by a competitive ELISA. The ELISA profile obtained from the haemolymph was very similar to mRNA content using the combined method of quantitative RT-PCR and Southern blot. Has-DTH-like immunoreactivity was 2–10-fold higher during the nondiapausing pupae than in the diapausing pupae. The results also support our idea that DTH play an important role for diapause termination. When diapause is terminated, some genes involved in the mechanisms that suppress development might be switched off and new sets of genes involved in initiating development might be switched on [33]. Thus, DTH gene expression at this stage from the SGs seems to be directly involved in diapause termination through DTH biosynthesis. Furthermore, the correlation between DTH gene expression and diapause termination provides additional evidence that DTH gene expression is a vital event in the molecular processes leading to diapause termination.

Hodek and Hodková [34] pointed out the hypothesis that diapause development is dependent on low temperature has been overgeneralized. In many species, a lower temperature is required for completion of diapause. One dispute on the relationship between diapause completion and low temperature concerns whether chilling actually accelerates diapause development or not. In our research, Has-DTH mRNA content and immunoreactivity both did not increase significantly when diapausing pupae were chilled at 4 °C for 40 days. This indicated that under the low temperature condition, environmental signal was accepted by brain, but not in SG. When the diapausing pupae were transferred from 4 to 25 °C, Has-DTH increased rapidly and largely in the SG or haemolymph and showed that Has-DTH gene expression is dependent on temperature as Bom-DH-PBAN gene expression [24]. The synthetic amidated Has-SGNP I had no effect on the diapausing pupae incubated at 22 °C and implied that biological activity of Has-DTH also directly correlate with the environmental temperature. However, it is still unknown how the environment information received in the diapausing pupae is stored in the brain and transmitted to control the DTH gene expression in the SG.

Many studies have been taken great headway on insect diapause which involves biochemical, molecular biological and endocrinological aspects. However, studies on diapause termination are poorly understood. The finding that DTH can break pupal diapause will shed a light on the mechanism of diapause termination. The pupal diapause termination of *H. assulta* by Has-DTH was another excellent model besides that of *H. armigera*. Considering the similar phenotypes of pupal diapause in insects, especially in lepidopteran insects, termination of pupal diapause and acceleration of pupal development might be the general function of Has-DTH.

Acknowledgements

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