ZOOLOGICAL SCIENCE **22**: 213–221 (2005)

Establishment of a Sandwich ELISA System to Detect Diapause Hormone, and Developmental Profile of Hormone Levels in Egg and Subesophageal Ganglion of the Silkworm, *Bombyx mori*

Norio Kitagawa[1], Kunihiro Shiomi[1,†], Kunio Imai[2], Teruyuki Niimi[1], Toshinobu Yaginuma[1*] and Okitsugu Yamashita[1,‡]

[1]*Laboratory of Sericulture and Entomoresources, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan*
[2]*Faculty of Bioresources, Mie University, Kamihama, Tsu 514-8507, Japan*

ABSTRACT—In the silkworm *Bombyx mori*, diapause hormone (DH) is produced in the female subesophageal ganglion (SG) and induces embryonic diapause by targeting developing ovaries. DH is processed from a precursor protein consisting of DH, pheromone biosynthesis activating neuropeptide (PBAN) and three other neuropeptides (SGNPs). Because these five neuropeptides share a common sequence, FXPRLamide, at the C-terminus, a direct and specific assay for DH itself is required in order to understand the profile of concentration changes. In this study, we produced a mouse monoclonal antibody (anti-DH[N] mAb) against the N-terminal region of DH and developed a sandwich enzyme-linked immunosorbent assay using the anti-DH[N] mAb and a rabbit polyclonal antibody against the C-terminus of DH. This procedure enabled us to specifically quantify the DH molecule at femtomolar levels (equivalent to 1/10 of SG). We then plotted DH levels in eggs and SGs during embryonic and post-embryonic development. DH was present in late-stage embryos that had been destined for the production of both diapause and nondiapause eggs. DH levels in SG gradually increased in both types during larval development and peaked at the early pupal stage. At the middle pupal stage, DH levels in SG and SG-brain complex decreased markedly in the diapause-egg producing type, thus indicating active release of DH into the hemolymph. From 5th instar larva to adult, no sexual differences in DH levels were observed in SGs or SG-brain complexes from diapause and nondiapause egg-producing types.

Key words: Diapause hormone, FXPRLamide neuropeptide family, bivoltinism, *Bombyx mori*, subesophageal ganglion, sandwich enzyme-linked immunosorbent assay

INTRODUCTION

Embryonic diapause in the silkworm *Bombyx mori* occurs immediately after mesoderm segmentation (Miya, 2003). Diapause is induced by a neuropeptide hormone (diapause hormone, DH) that is synthesized in the female subesophageal ganglion (SG) and targets developing ovaries (Yamashita and Hasegawa, 1985; Yamashita, 1996).

On the other hand, in the case of the *Bombyx* bivoltine race, offspring diapause is determined by the mother's experience during embryonic development. For example, when eggs of the bivoltine race (Daizo) are incubated at 25°C, the resultant female moths can lay diapause eggs. In contrast, incubation at 15°C during mother's embryonic stage causes the resultant moths to lay nondiapause eggs (Watanabe, 1924; Xu *et al.*, 1995b; Morita *et al.*, 2003). If eggs are incubated at intermediate temperatures, such as 20°C, continuous illumination or darkness makes the moths produce diapause or nondiapause eggs, respectively (Kogure, 1933; Morita *et al.*, 2003). Furthermore, the brain is believed to regulate whether DH is released from the SG into the hemolymph, although DH is present in the SG of nondiapause-egg producers (Fukuda, 1952; Sonobe *et al.*, 1977; Matsutani and Sonobe, 1987; Shimizu *et al.*, 1997; Ichikawa, 2003). These results indicate that signals regarding the mother's embryonic experience are received by and integrated in the brain, which then regulates DH biosynthe-

* Corresponding author. Phone: +81-52-789-4038;
Fax : +81-52-789-4036;
E-mail: yaginut@agr.nagoya-u.ac.jp
† Present address: Faculty of Textile Science and Technology, Shinshu University, Ueda, Nagano 386-8567, Japan
‡ Present address: Chubu University, Kasugai, Aichi 487-8501, Japan

sis in the SG and/or DH release from the SG into hemolymph. Therefore, we would like to understand the molecular processes connecting the reception of environmental signals during embryonic development with the production of DH in the SG and the release of DH from the SG into hemolymph during pupal development. For this purpose, we must first develop methods to detect and quantify the DH molecule itself.

DH is a 24-amino acid peptide amide belonging to FXPRLamide neuropeptide family (Fig. 1A; Imai *et al.*, 1991; Sato *et al.*, 1992, 1993). DH is processed from a precursor protein that is composed of DH, pheromone biosynthesis activating neuropeptide (PBAN) and three SG neuropeptides (SGNPs) (Kawano *et al.*, 1992; Sato *et al.*, 1993; Xu *et al.*, 1995a). These five neuropeptides share a common amino-acid sequence at the C-terminus, FXPRLa (See Fig. 1A). Therefore, this gene is now called *DH-PBAN* (Xu *et al.*, 1995a; Yamashita, 1996). To date, the DH-producing neurosecretory cells in the SG and the DH transport route were identified by Ichikawa *et al.* (1995) using a mouse polyclonal antibody against the N-terminal 12 amino-acid sequence of DH and by Sato *et al.* (1998) using a rabbit anti-FXPRLa peptide antibody that was not able to distinguish between other FXPRLa peptides, such as PBAN and SGNPs (Sato *et al.*, 1998). In order to examine DH levels in various tissues, such as the SG, and hemolymph, we had to produce antibodies that were specific to DH and had very high reactivity.

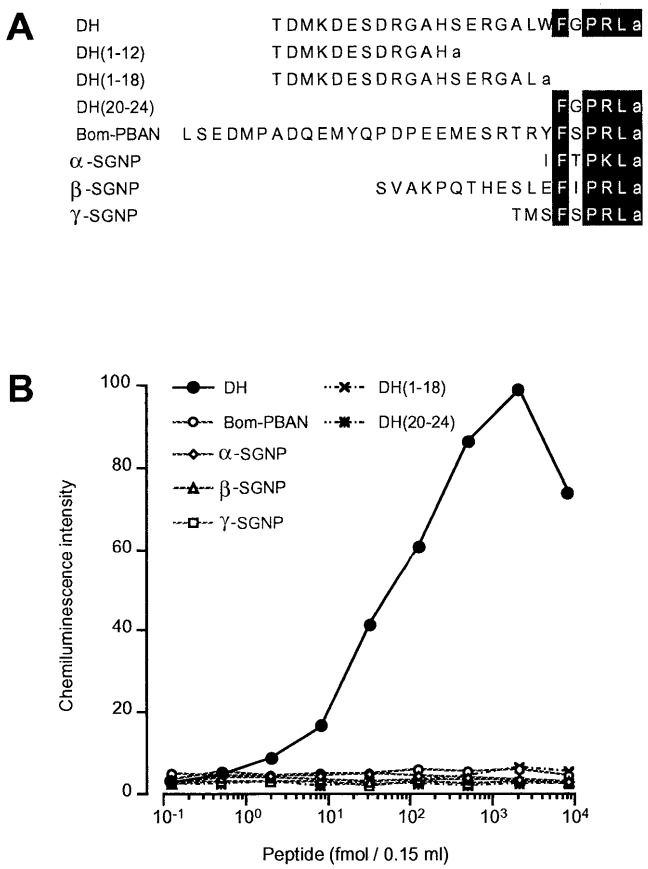

Fig. 1. Quantification of diapause hormone (DH) by sandwich ELISA. (A) Synthetic peptides used here for sandwich ELISA. Gray regions depict conserved FXPRLamide sequences. Lowercase "a" indicates C-terminal amidation. DH and DH (1-12) were originally used as antigens for anti-DH[C] IgG and anti-DH[N] mAb, respectively. (B) Dose-response curve of sandwich ELISA for specific quantification of DH. Indicated amounts of various DHs, Bom-PBAN, and α-, β- and γ-SG neuropeptides (SGNPs) were added to each well. The results are shown as the chemiluminescence intensity relative to the highest value (100%). Chemiluminescence intensity expressed by sandwich ELISA (See Materials and Methods) was detected by Lumi Imager^{TM} F1. In this system, only DH is quantified.

In this study, we produced a mouse monoclonal antibody (anti-DH[N] mAb) recognizing the N-terminal area of DH and developed a sandwich enzyme-linked immunosorbent assay (ELISA) using this mAb and a previously produced rabbit polyclonal antibody against the C-terminal area of DH. This sandwich ELISA system allowed the quantification of the DH molecule itself at femtomolar levels (equivalent to 1/10 of SG). We then followed the changes in DH levels in eggs and SGs during larval-pupal-adult development.

MATERIALS AND METHODS

Animals

A bivoltine race (Daizo) of the silkworm *Bombyx mori* was used because offspring diapause in this race depends on the incubation temperature during mother's embryonic stage. One group of eggs was incubated at 25°C with continuous illumination (diapause-egg producer), and the other was incubated at 15°C in continuous darkness (nondiapause-egg producer). Both the larvae were reared on an artificial diet (Silk-Mate 1M, Nihon Nosan Kogyo Co., Yokohama, Japan) until the 4th instar larval stage, after which 5th instar larvae were reared on fresh mulberry leaves at 25–27°C without specific photoperiod control. Pupae were kept at 25°C until adult emergence. After copulation, the female moths were allowed to lay eggs, which were then kept at 25°C. The diapause state of the laid eggs was confirmed 2 weeks after oviposition, when larvae had already hatched from all nondiapause eggs. In this race, diapause- and nondiapause-egg producers laid 100% diapause or 100% nondiapause eggs, respectively (Morita *et al.*, 2003). To prevent entrance into diapause, the diapause eggs were treated with HCl solution (specific gravity 1.075 at 15°C) at 25°C for 90 min at around 20 h after oviposition (Morita *et al.*, 2003).

Preparation of DH and other peptides

Bombyx PBAN (Bom-PBAN) was kindly provided by Dr. S. Matsumoto (RIKEN, Wako, Japan). Other peptides were synthesized as described previously (Sato *et al.*, 1998). A peptide corresponding to the first 12 amino-acid residues from the N-terminus of DH, Thr-Asp-Met-Lys-Asp-Glu-Ser-Asp-Arg-Gly-Ala-His [DH (1-12)], conjugated with bovine serum albumin (BSA) via addition of Cys was used as the antigen to produce the monoclonal antibody. The synthetic peptides used in this study are listed in Fig. 1A.

Preparation of antibodies

The procedure for preparation of rabbit antiserum against synthetic DH was as described previously (Shiomi *et al.*, 1994; Sato *et al.*, 1998). This antiserum is able to recognize the FXPRLamide sequence at the C-terminal side of members of FXPRLamide neuropeptide family including DH (Sato *et al.*, 1998). The IgG fraction was purified from this antiserum using AffinityPak^{TM} Protein A Col-

umns (Pierce, IL), dialyzed against PBS (20 mM sodium phosphate, pH 7.2, containing 130 mM NaCl) and stored at a concentration of 0.96 mg/ml at –20°C. The purified antibody is hereafter referred to as anti-DH[C] IgG.

The mouse monoclonal antibody against the N-terminal region of DH was produced using BSA-conjugated-synthetic peptide [DH (1-12)] according to the general protocol (Kitayama Labes Co. Ltd., Nagano, Japan). This monoclonal antibody was designated as anti-DH[N] mAb.

DH extraction

SGs or SG-Brain complexes of 3–15 animals were dissected in cold PBS on ice. Tissues were pooled in 250 µl of ice-cold extraction buffer (supernatant after centrifugation of 5% skim milk solution in PBS at 21,130×*g* for 10 min), and a given amount of DH was added, if necessary. After sonication for 1 min, the tissue suspension was boiled for 5 min and cooled on ice for 10 min. Aliquots of the supernatant after centrifugation at 21,130×*g* (corresponding to 0.08 to 6 SGs) were used for the assay. For the egg stage, 50 mg of eggs plus a given amount of DH or no DH were respectively homogenized in 500 µl of the ice-cold extraction buffer. The homogenate was boiled for 10 min, and then cooled on ice for 10 min. The sample was further centrifuged at 21,130×*g* for 10 min at 4°C, and 100 µl of the supernatant (corresponding to 20 eggs) was used for the assay.

Sandwich ELISA

Anti-DH[C] IgG was added to each well of a 96-well micro-titer plate (Costar #3922; Corning Inc., NY) in 750 ng aliquots, and the plate was then incubated overnight at 4°C. After washing five times with wash buffer (PBS containing 0.1% Tween-20), 150 µl of PBS containing 5% skim milk was added to the wells and then incubated for 1 h at room temperature. After washing twice with wash buffer, either synthetic standard DH [DH (1-24)] or 100 µl of the test sample diluted with extraction buffer was added to the wells. Then, mouse ascitic fluid (50 µl) containing anti-DH[N] mAb, diluted to 1:2500 with PBS containing 0.1%Tween-20 and 2% skim milk, was added to each well, and the plate was incubated overnight at 4°C. Wells were washed three times with wash buffer, and filled with 150 µl of horseradish peroxidase (HRP)-labeled F(ab')$_2$ goat anti-mouse IgG (H+L) antibody (Zymed #62-6320; Zymed Laboratories Inc., CA), diluted to 1:4000 with PBS containing 0.1% Tween-20, and the plate was further incubated for 3 h at room temperature. During each incubation step, the plate was sealed with plate seal (Sumitomo Bakelite Co., Tokyo) in order to prevent evaporation of the well contents. Finally, the wells were washed three times with wash buffer, and were filled with 150 µl of substrate (Super Signal ELISA Femto Maximum Sensitivity Substrate; Pierce, IL) that was pre-incubated at 37°C for 5 min, and the intensity of chemiluminescence was then measured by Lumi Imager™ F1 (Roche, Mannheim).

Statistics

Statistical analysis was carried out using the SPSS software package (Version 10).

RESULTS

Sandwich ELISA for specific quantification of DH

As described previously, the *DH-PBAN* gene codes for a precursor protein, from which DH, PBAN, and α-, β- and γ-SG neuropeptides (SGNPs) are processed (Sato *et al.*, 1993). They share a C-terminal FXPRLamide sequence (Fig.1A). When the full-length DH (1-24) with amidation at the C-terminus was used as an antigen to make antibodies against DH, we obtained only polyclonal antibodies (anti-DH[C] IgG) that are able to recognize the FXPRLa area but are not able to distinguish DH from other FXPRLa peptides (Sato *et al.*, 1998). We therefore changed the immunogen to a peptide containing the N-terminal area of [DH(1-12)] and injected the new peptide-conjugated BSA into mice to obtain monoclonal antibodies. After several efforts, we were able to isolate a monoclonal antibody (anti-DH[N] mAb) specific to DH (1-12). To detect DH with high sensitivity, we developed a sandwich ELISA system using the anti-DH[N] mAb and a rabbit anti-DH[C] IgG. In this system, when each FXPRLa peptide in Fig. 1A was trapped with anti-DH[C] IgG immobilized on the bottom surface of micro-titer plate wells, full-length DH was expected to be recognized by anti-DH[N] mAb, which is bound with anti-mouse IgG antibody conjugated to HRP. As shown in Fig.1B, using this system DH (1-24) was specifically detected in the range of 1 fmol to 2 pmol. In contrast, PBAN, and α-, β- and γ-SGNPs up to 10 pmol did not give chemiluminescence signals. The absence of appreciable signals with DH fragments [DH (1-18) or DH (20-24)] lacking hexapeptide (WFGPRLa) at the C-terminus or nonadecapeptide at the N-terminus demonstrated that this system could detect only full-length DH (1-24 plus amide).

Quantification of DH in tissue extract

When chemiluminescence intensities in this ELISA were plotted against 7 fmol to 2 pmol of DH (1-24) in semi-logarithmic graph, a regression line ($y=1.392 \log(x) - 0.408$; $r^2=0.995$) was found (Fig. 2A). Next, to test the application of this system to tissue extracts, extracts from SGs of 5th instar larvae at different concentrations were subjected to this assay (Fig. 2A). When SG extracts over a range of 0.08 to 1.0 SG equivalents were applied, a largely regressed line ($y=1.461 \log(x) - 0.362$; $r^2=0.905$) was observed in parallel with that for synthetic DH. We concluded that SG extract did not contain substances disturbing the sandwich ELISA reaction at relative chemiluminescence intensities of 25 to 90% (Fig. 2A).

For correction of the efficiency of DH extraction from tissues and the influence on ELISA by substances in the tissue extract, known amounts of synthetic DH were added to the pools of dissected SGs or the eggs, and these were then subjected to the extracting or homogenizing procedures. When the amounts of DH in the tissues quantified by ELISA were plotted against the amounts of synthetic DH added, respective regression lines with different slopes (0.907 for SGs and 0.679 for eggs) were observed (Fig. 2B). Because we could assume that the slope of each line was directly proportional to the detection efficiency of DH from extracts of SGs or eggs, we divided the amounts of originally quantified DH by the slope of each regression line for SGs or eggs. For practical assay of DH in numerous samples from the egg to adult stages, we independently prepared triplicate samples to which known amounts of synthetic DH were added, and estimated the averaged correction factor using

the following equation:
correction factor = ([B] – [A]) / [C]
where [A], [B], and [C] represent the amount of DH in the tissue extract, the amount of DH in the tissue extract plus the known amount of synthetic DH added, and the amount of synthetic DH added, respectively.

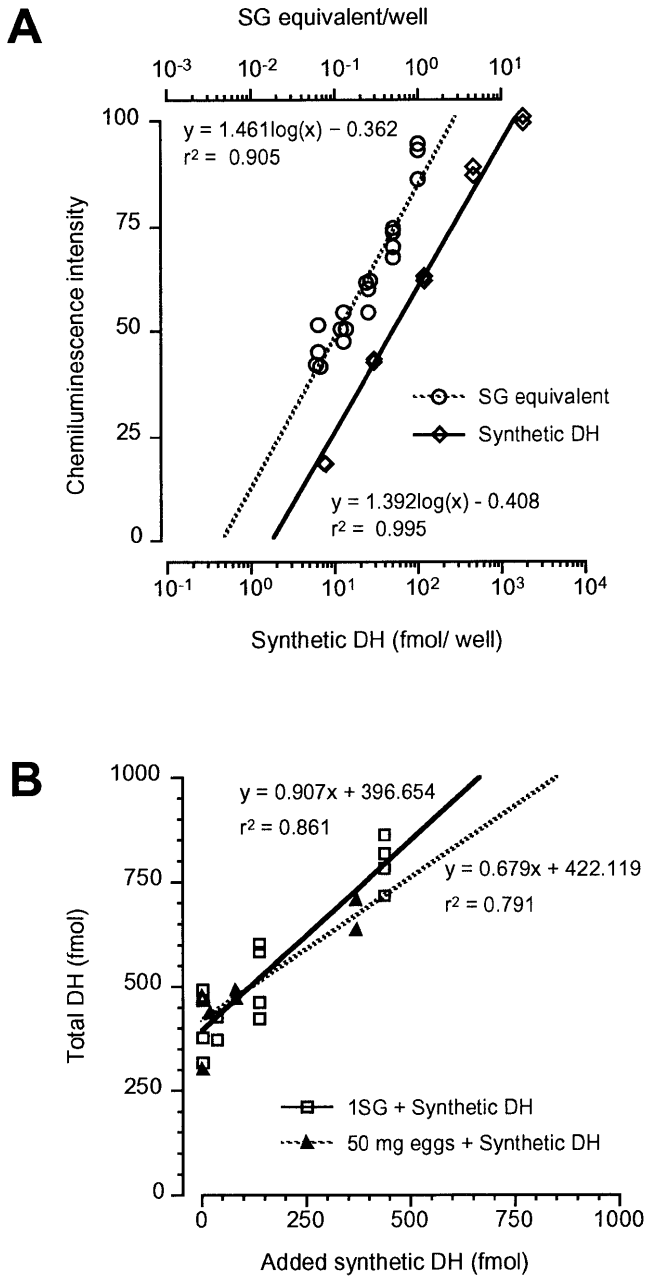

Fig. 2. Quantification of DH in extracts from SGs and eggs by sandwich ELISA. (A) Relationship between the chemiluminescence intensity and the concentrations of extracts from SG (open circles) and synthetic DH (open diamonds) that are expressed as logarithmic plots. The results are shown as the relative chemiluminescence intensity as in Fig. 1B. SGs were used from 1-day old 5th instar larvae in diapause-egg producers. (B) Detection efficiency of DH from extracts of SGs (open squares) or eggs (closed triangles). To one SG or eggs (50 mg), indicated amounts of synthetic DH were added before the extraction. SGs or eggs were respectively used from 3-day old 3rd instar larvae in diapause-egg producers or from the stage of 90% embryonic development in diapause-egg producers.

Developmental profile of DH levels during embryonic development of *Bombyx* diapause- and nondiapause-egg producers

As described above, we established the sandwich ELISA system to detect DH in eggs or SGs. Therefore, we first examined DH levels during embryonic development. Here, we compared the DH levels in two types of egg from the bivoltine race (Daizo). Xu *et al.* (1995b) reported that *DH-PBAN* mRNA expression was activated in the late embryonic stage in diapause-egg producers, while Morita *et al.* (2003) proposed that *DH-PBAN* mRNA expression was activated near larval hatching in both diapause- and nondiapause-egg producers.

We first treated 20-h old diapause eggs of Daizo with HCl solution in order to prevent entrance into diapause, and we then incubated the eggs for further embryonic development under the following conditions: to give diapause-egg producers, eggs were incubated continuously at 25°C with constant illumination (larval hatching occurred after 9 days); to give nondiapause-egg producers, eggs were incubated at 25°C for a further 2 days with constant illumination and were then incubated at 15°C in constant darkness (larval hatching

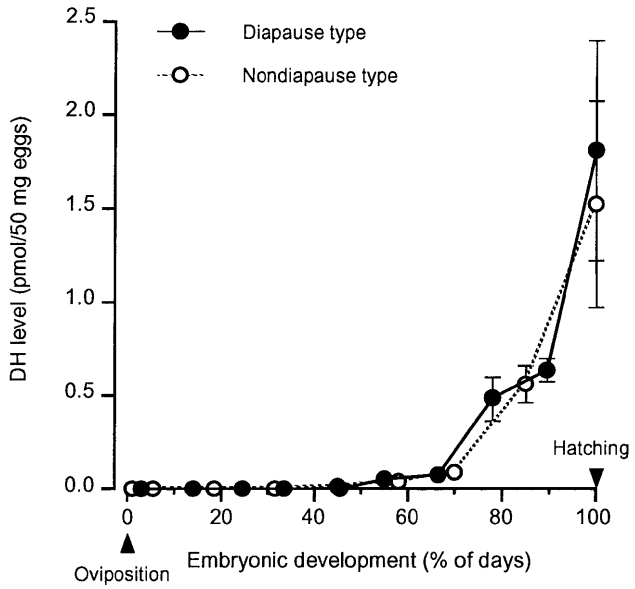

Fig. 3. Profile of DH levels during embryonic development. Diapause eggs of Daizo (bivoltine race) were soaked with HCl solution (specific gravity 1.075 at 15°C) at 20 h after oviposition to prevent entrance into diapause. One group was incubated at 25°C under continuous illumination to give diapause-egg producers (closed circles). Larval hatching took 9 days. The other group was incubated at 25°C under illumination for 2 days and then at 15°C under darkness to give nondiapause-egg producers (open circles). The egg duration was 21 days. Here, the entire period from oviposition to larval hatching is expressed as 100%. Eggs were homogenized and extracted with boiling water, and then the supernatant was used for DH measurement. Each point represents an averaged value for 5 samples±SD.

occurred after 21 days). In order to compare developmental stages between the two types of egg, DH levels were plotted against the relative developmental time, with 100% as the entire period from oviposition to larval hatching (Fig. 3).

DH became clearly detectable at around 80% complete embryonic development in both diapause- and nondiapause-egg producers. DH levels increased toward larval hatching and reached about 1.6 pmol/50 mg of eggs in both types; this value corresponds to about 16 fmol/egg (wet weight of one egg is about 0.5 mg). We were not able to identify significant differences in DH levels between diapause- and nondiapause-egg producers. These changing patterns in DH levels between the two types of egg were almost the same as those in *DH-PBAN* mRNA levels reported by Morita *et al.* (2003).

Developmental profile of DH levels in SG during post-embryonic development of *Bombyx* diapause- and non-diapause-egg producers

Larval and pupal development of nondiapause-egg producers progressed faster than that of diapause-egg producers. In the diapause-egg producers, adult emergence started 32 days after larval hatching, but started 27 days after larval hatching in nondiapause-egg producers. Therefore, in Fig. 4, the developmental period of nondiapause-egg producers was expanded for comparison with that of diapause-egg producers. From the 1st to 4th instar, larvae were not sexed. From the 5th instar larva to adult, SGs were dissected from males and females of the same number. From the middle pupal stage to adult, it was difficult to isolate only the SG because the SG is fused with the brain.

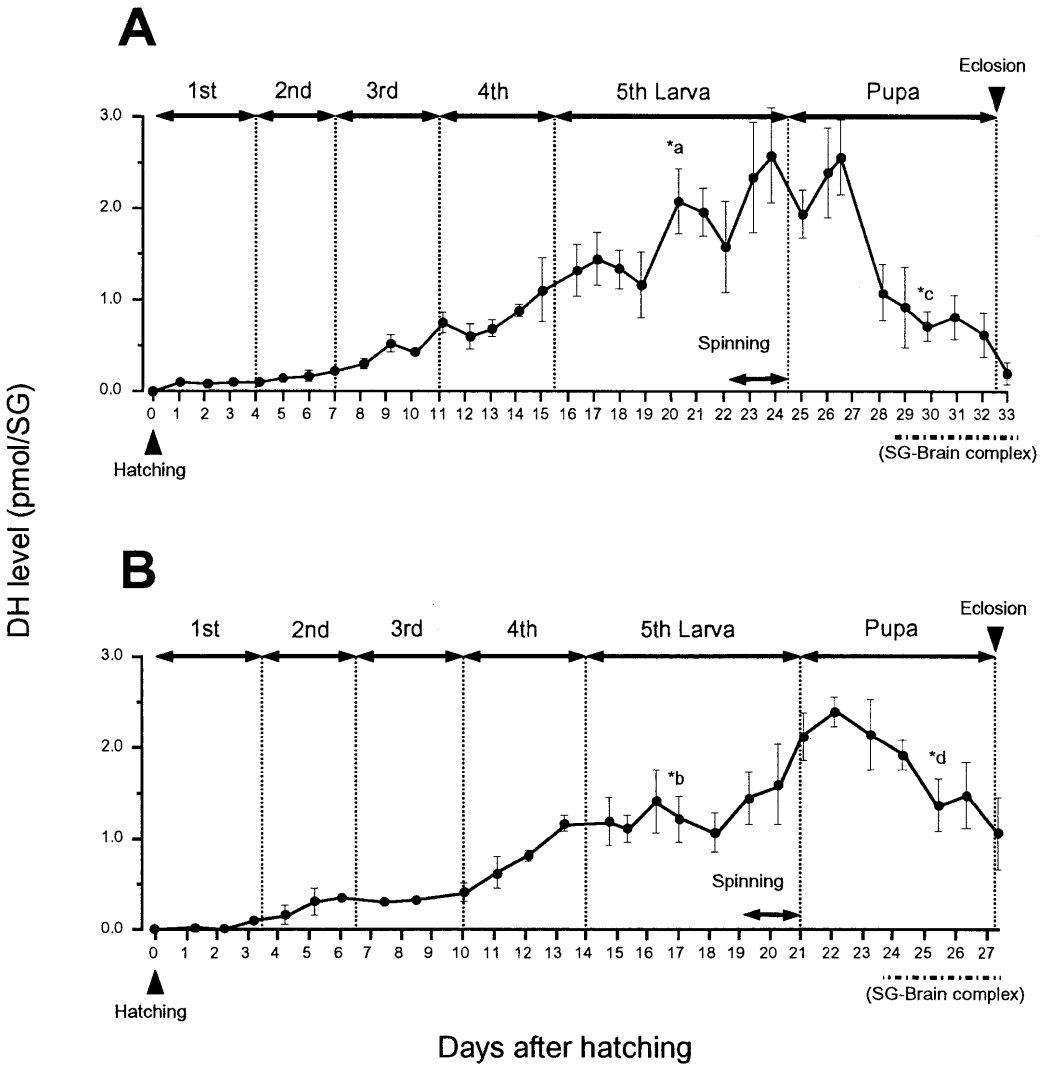

Fig. 4. Profile of DH levels in SG during post-embryonic development. From the 1st to 4th instar, larvae were not sexed. After the 5th instar, silkworms were mixed at a sex ratio of 1:1. Next, 3 to 15 SGs were extracted with boiling water after sonication, and the supernatant was used for DH measurement. In the late pupal and adult stages, SG-Brain complexes were used (dashed underlines) because the SG and brain were fused. Each point represents an averaged value for 3 samples±SD. DH levels in the SG or SG-Brain complex of diapause- (A) or nondiapause- (B) egg producers. In (A) and (B), DH levels were 0.0197 and 0.0176 pmol/SG at hatching, respectively. "*a and *b" and "*c and *d" were significantly different (p=0.002) by non-parametric (Mann-Whitney) test.

During these stages, SG and brain (SG-Brain complex) were dissected and used as starting materials.

Both types showed similar profiles in DH levels during larval development, except that levels in diapause-egg producers were slightly higher in the late 3rd instar larval stage, and that two peaks were observed at the late 5th instar larval stage in diapause-egg producers (Fig. 4AB). A clear contrast between the diapause and nondiapause types was that DH levels in the SG or SG-Brain complex from the diapause type steeply declined in the middle pupal stage, thus suggesting active release of DH from the SG into hemolymph. Around the middle pupal stage, developing ovaries are known to develop increased sensitivity to DH for induction of diapause eggs (Hasegawa, 1963; Yamashita and Hasegawa, 1966; Yamashita and Hasegawa, 1970).

Although the target organ of DH is the female ovary as previously described, the physiological roles of DH in males are not known (Ichikawa and Suenobu, 2003). In Fig. 5, DH levels in the SG were compared between males and females in both types of animals. We were not able to identify large differences in DH levels between males and females. The two additional peaks during development of 5th instar larva and the steep decline in the middle pupal stage were also observed in males of diapause type.

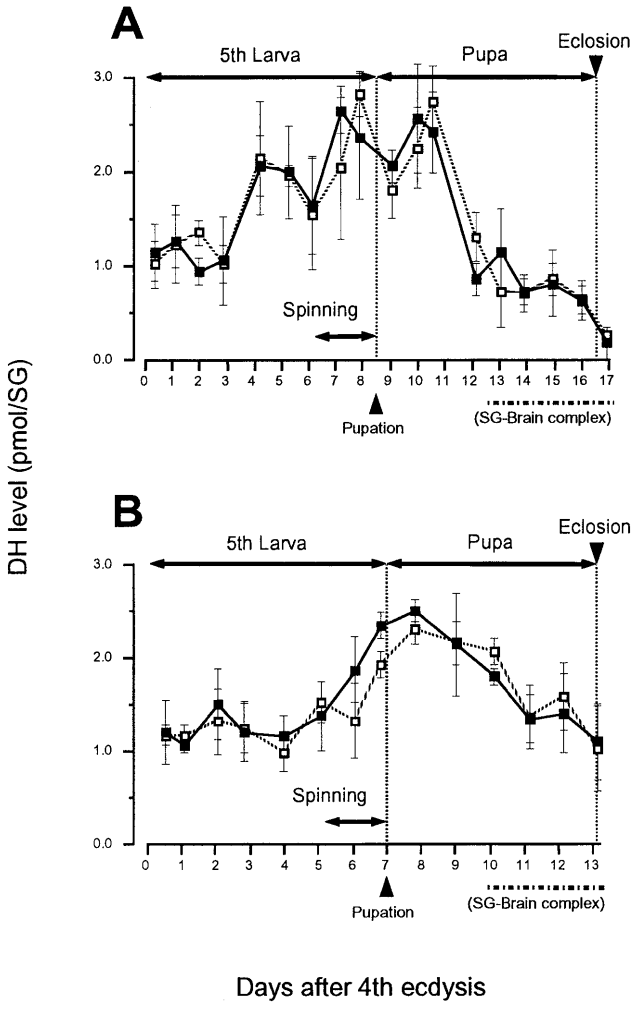

Fig. 5. Profile of DH levels in male or female SGs during 5th larval-pupal-adult development. DH levels in SGs or SG-Brain complexes of diapause- (A) or nondiapause- (B) egg producers. SGs or SG-Brain complexes of females (closed squares) and males (open squares) were extracted as described in Fig. 4. During the late pupal and adult stages, SG-Brain complexes were used (dashed underline). Each point represents an averaged value for 3 samples±SD.

DISCUSSION

In this study, we isolated a novel mouse monoclonal antibody (anti-DH[N] mAb) recognizing the N-terminal area of DH [DH(1-12)], and together with the use of a rabbit polyclonal antibody (anti-DH[C] IgG) specific to the C-terminal area of DH (FXPRLa area; Sato *et al.*, 1998), we established a sandwich ELISA system that is able to quantify DH specifically at the femtomolar level (equivalent to 1/10 of SG; Figs. 1 and 2). This system is more sensitive when compared with other immunoassays for insect neuropeptides, such as ecdysis-triggering hormone (Kingan *et al.*, 1997), FLRFamide (Kingan *et al.*, 1997), diuretic hormone (Audsley *et al.*, 1997), allatostatin (Yu *et al.*, 1993; Audsley *et al.*, 1998) or adipokinetic hormone (Goldsworthy *et al.*, 2002), but is less sensitive than the time-resolved fluoroimmunoassay for prothoracicotropic hormone, which is able to detect attomolar levels (Mizoguchi *et al.*, 2001).

Using this ELISA system, DH levels were first examined in the course of *Bombyx* embryonic development, but significant differences in DH levels were not observed between two types of bivoltine eggs, which had been incubated at 25°C under illumination or at 15°C in darkness. This is not consistent with the *DH-PBAN* mRNA levels shown previously by Xu *et al.* (1995b). However, recent results reported by Morita *et al.* (2003) concerning the quantification of *DH-PBAN* mRNA using Northern hybridization and real-time quantitative PCR clearly indicate that there are no differences in *DH-PBAN* mRNA levels between both types of egg. When embryonic head parts from eggs incubated at 15°C under continuous darkness are implanted into 0-day old pupae of a nondiapause race (N_4), the N_4 female moths laid diapause eggs, thus suggesting that these embryonic head parts produce significant amounts of DH (Morita *et al.*, 2003). These results are in agreement with the present data. We thus conclude that there is no relationship between temperature/photoperiod and DH production during embryonic development. The present data clearly demonstrate that along with larval differentiation, SG becomes to be able to produce DH independently of egg incubation conditions.

We then followed DH levels in the SG during post-embryonic development. Both diapause and nondiapause types showed similar developmental profiles in DH levels in the SG during the larval and early pupal stages, and the DH levels increased with larval age. From the middle to the late larval stages, DH levels in the SG of diapause-egg producers appear to be higher than those in nondiapause-egg pro-

ducers (Fig. 4). Although SGs in both types maintain higher amounts of DH during the early pupal stage, DH levels in the SG of diapause-egg producers decrease during the middle pupal stage (Figs. 4 and 5). When exogenous DH is injected into pupae of a nondiapause race (N_4) or into pupae from which the SGs were removed at day 0 pupa, developing ovaries at the middle pupal stage are most sensitive to DH for the production of diapause eggs (Hasegawa, 1963; Yamashita and Hasegawa, 1966; Yamashita and Hasegawa, 1970). Therefore, the rapid decline of DH levels in the SG or SG-Brain of diapause-egg producers (Figs. 4 and 5) may indicate the active release of DH into hemolymph during the middle pupal stage.

The present results differ somewhat from those reported by Sonobe *et al.* (1977), in which DH levels were estimated based on the amount of 3-hydroxykynurenine (precursor of ommochrome pigments in serosa cells of diapause eggs) accumulated in ovaries after an SG extract was injected into pupae; DH increases 3-hydroxykynurenine levels in ovaries (Sonobe *et al.*, 1977). According to Sonobe *et al.* (1977), DH levels in the SG were constant in 5th instar larva, increased from 0.5 to 6–7days after pupation, and then declined toward 1 day after adult emergence in both sexes of nondiapause-egg producers and male diapause-egg producers, whereas they decreased from 3 to 5 days after pupation without an increase in female diapause-egg producers. Although we cannot clearly explain the reason for the discrepancy between the present results and those of Sonobe *et al.* (1977), the SG extract (Sonobe *et al.* 1977) may contain substances, such as PBAN, SGNPs and other FXPRLa, that are able to stimulate the accumulation of 3-hydroxykynurenine in ovaries. Toward adult emergence, the decrease in DH levels in the female SG-Brain complex irrespective of diapause or nondiapause egg production (Figs. 4 and 5) may be related to PBAN release, thus suggesting that DH might be released together with PBAN.

The *DH-PBAN* gene is expressed in three clusters of neurosecretory cells [mandibular (Md), maxillary (Mx) and labial (Lb) cells] in the SG (Sato *et al.*, 1994). These cells were identified by immunohistochemical staining using antisera recognizing DH, PBAN and FXPRLa peptides, respectively (Ichikawa *et al.*, 1995; Sato *et al.*, 1998). The functional differentiation of the cells was suggested in a surgical ablation experiment; Lb and Mx/Md cells are responsible for induction of diapause eggs and for activation of sex pheromone production, respectively (Ichikawa *et al.*, 1996). Recently, a difference in firing activity between diapause and nondiapause types was found only in Lb cells (Ichikawa, 2003; Ichikawa and Kamimoto, 2003), thus suggesting that Lb cells are related to regulation of DH release. The present results on the sudden decline of DH levels in the SG of diapause-egg producers during the middle pupal stage may reflect this firing of Lb cells.

During the 5th larval and pupal stages, sexual differences in DH levels were not observed in the SGs of diapause- and nondiapause-egg producers (Fig. 5). This suggests that the release of DH from the male SG is regulated by the same mechanism as the female SG, although the DH signal is only visualized in females with ovaries. When the ovarian disk is implanted into male diapause-egg producers, the mature eggs show the properties of diapause eggs, such as enhanced accumulation of 3-hydroxykynurenine and glycogen (Hasegawa, 1952; Hasegawa and Yamashita, 1965). When these matured eggs are activated parthenogenetically, they enter diapause (Yamashita and Irie, 1980). Recently, Ichikawa and Suenobu (2003) used the parabiotical connection experiment to confirm that pupal hemolymph of male diapause-egg producers is able to enhance accumulation of 3-hydroxykynurenine in ovaries of female pupae from which the SG was removed. These results clearly indicate that there are significant levels of DH in the pupal hemolymph of male diapause-egg producers. This is also supported by the recent measurement of firing activity in DH-producing cells, in which Ichikawa and Suenobu (2003) found similar activities in male cells. What is the function of DH in male systems? Because a *Bombyx* DH receptor was recently identified in developing ovaries (Homma *et al*, 2004), by determining where DH receptors are expressed in males, the target organ may be identified.

In order to confirm whether the sudden decline in DH levels in SG is indicative of release of DH from the SG into hemolymph, DH levels in the hemolymph of both sexes of diapause- and nondiapause-egg producers must be measured. Unfortunately, when we prepared hemolymph samples using the same extraction method as for SGs and subjected them to this sandwich ELISA, we were not able to detect DH in hemolymph with synthetic DH added, which suggests that other substances in the hemolymph interfere strongly with the ELISA. When we extracted DH from 100 μl of hemolymph using a small column of GL-Pak PLS (GL Sciences Inc., Tokyo) and used this DH fraction, we were able to obtain significant chemiluminescence signals after sandwich ELISA. We are currently refining the conditions for column extraction of hemolymph in order to improve reproducibility. By using a combination of sandwich ELISA and column extraction, we hope to quantify the amount of DH in hemolymph.

ACKNOWLEDGMENTS

We would like to thank Prof. M. Kobayashi and Assoc. Prof. M. Ikeda for productive discussions. We also thank Dr. S. Matsumoto, RIKEN, for providing Bom-PBAN. This study was supported in part by a grant from the Ministry of Education, Science, Sports and Culture of Japan (08276103; 16208007), by a Grant-in-Aid (BDP) from the Ministry of Agriculture, Forestry and Fisheries, and by the Research for the Future Program of the Japan Society for the Promotion of Science (JSPS-RFTF99L01203).

REFERENCES

Audsley N, Goldsworthy GJ, Coast GM (1997) Circulating levels of *Locusta* diuretic hormone: The effect of feeding. Peptides 18:

59–65
Audsley N, Weaver RJ, Edwards JP (1998) Enzyme linked immunosorbent assay for *Manduca sexta* allatostatin (Mas-AS), isolation and measurement of Mas-AS immunoreactive peptide in *Lacanobia oleracea*. Insect Biochem Mol Biol 28: 775–784
Fukuda, S (1952) Function of the pupal brain and subesophageal ganglion in the production of non-diapause eggs and diapause eggs in the silkworm. Annot Zool Japon 25: 149–155
Goldsworthy GJ, Kodrik D, Comley R, Lightfoot M (2002) A quantitative study of adipokinetic hormone of the firebug, *Pyrrhocoris apterus*. J Insect Physiol 48: 1103–1109
Hasegawa K (1952) Studies on the voltinism in the silkworm, *Bombyx mori* L. with special reference to the organs concerning determination of voltinism. J Fac Agric Tottori Univ 1: 83–124
Hasegawa K (1963) Studies on the mode of action of the diapause hormone in the silkworm, *Bombyx mori* L. I. The action of the diapause hormone injected into pupae of different ages. J Exp Biol 40: 517–529
Hasegawa K, Yamashita O (1965) Studies on the mode of action of the diapause hormone in the silkworm, *Bombyx mori* L. VI. The target organ of the diapause hormone. J Exp Biol 43: 271–277
Homma T, Watanabe K, Kataoka H, Imai K, Niimi T, Yamashita O, Yaginuma T (2004) Cloning and identification of cDNA encoding a Bombyx *receptor* for diapause hormone. Proc 74th ann meeting Japan Soc Sericult Sci 74: 60
Ichikawa T (2003) Firing activities of neurosecretory cells producing diapause hormone and its related peptides in the female silkmoth, *Bombyx mori*. I. Labial cells. Zool Sci 20: 971–978
Ichikawa T, Hasegawa K, Shimizu I, Katsuo K, Kataoka H, Suzuki A (1995) Structure of neurosecretory cells with immunoreactive diapause hormone and pheromone biosynthesis activating neuropeptide in the silkworm, *Bombyx mori*. Zool Sci 12: 703–712
Ichikawa T, Kamimoto S (2003) Firing activities of neurosecretory cells producing diapause hormone and its related peptides in the female silkmoth, *Bombyx mori*. II. Mandibular and maxillary cells. Zool Sci 20: 979–983
Ichikawa T, Shiota T, Shimizu I, Kataoka H (1996) Functional differentiation of neurosecretory cells with immunoreactive diapause hormone and pheromone biosynthesis activating neuropeptide of the moth, *Bombyx mori*. Zool Sci 13: 21–25
Ichikawa T, Suenobu A (2003) Firing activities of diapause hormone producing cells in the male silkmoth, *Bombyx mori*. Zool Sci 20: 957–962
Imai K, Konno T, Nakazawa Y, Komiya T, Isobe M, Koga K, Goto T, Yaginuma T, Sakakibara K, Hasegawa K, Yamashita O (1991) Isolation and structure of diapause hormone of the silkworm, *Bombyx mori*. Proc Jpn Acad 67B: 98–100
Kawano T, Kataoka H, Nagasawa H, Isogai A, Suzuki A (1992) cDNA cloning and sequence determination of the pheromone biosynthesis activating neuropeptide of the silkworm, *Bombyx mori*. Biochem Biophysic Res Commun 189: 221–226
Kingan TG, Gray W, Zitnan D, Adams ME (1997) Regulation of ecdysis-triggering hormone release by eclosion hormone. J Exp Biol 200: 3245–3256
Kingan TG, Zitnan D, Jaffe H, Beckage NE (1997) Identification of neuropeptide in the midgut of parasitized insects: FLRFamides as candidate paracrines. Mol Cell Endocrinol 133: 19–32
Kogure M (1933) The influence of light and temperature on certain characters of the silkworm, *Bombyx mori*. J Fac Agric Kyusyu Imp Univ 4: 1–93
Matsutani K, Sonobe H (1987) Control of diapause-factor secretion in the silkworm, *Bombyx mori*: the roles of the protocerebrum and tritocerebrum. J Insect Physiol 33: 279–285
Miya, K (2003) The early embryonic development of *Bombyx mori* – An ultrastructural point of view-, Ed by T Yaginuma, K Suzuki, Gendaitosho, Sagamihara, 211 p
Mizoguchi A, Yasutaka O, Hosoda K, Ishibashi J, Kataoka H (2001) Developmental profile of the changes in the prothoracicotropic hormone titer in hemolymph of the silkworm *Bombyx mori*: correlation with ecdysteroid secretion. Insect Biochem Mol Biol 31: 349–358
Morita A, Niimi T, Yamashita O (2003) Physiological differentiation of DH-PBAN-producing neurosecretory cells in the silkworm embryo. J Insect Physiol 49: 1093–1102
Sato Y, Ikeda M, Yamashita O (1994) Neurosecretory cells expressing the gene for common precursor for diapause hormone and pheromone biosynthesis-activating neuropeptide in the suboesophageal ganglion of the silkworm, *Bombyx mori*. Gen Comp Endocrinol 96: 27–36
Sato Y, Nakazawa Y, Menjo N, Imai K, Komiya T, Saito H, Shin M, Ikeda M, Sakakibara K, Isobe M, Yamashita O (1992) A new diapause hormone molecule of the silkworm, *Bombyx mori*. Proc Jpn Acad 67B: 75–79
Sato Y, Oguchi M, Menjo N, Imai K, Saito H, Ikeda M, Isobe M, Yamashita O (1993) Precursor polyprotein for multiple neuropeptides secreted from the suboesophageal ganglion of the silkworm *Bombyx mori*: Characterization of the cDNA encoding diapause hormone precursor and identification of additional peptides. Proc Natl Acad Sci USA 90: 3251–3255
Sato Y, Shiomi K, Saito H, Imai K, Yamashita O (1998) Phe-X-Pro-Leu-NH2 peptide producing cells in the central nervous system of the silkworm, *Bombyx mori*. J Insect Physiol 44: 333–342
Shiomi K, Ishida Y, Ikeda M, Sato Y, Saito H, Imai K, Isobe M, Yamashita O (1994) Induction of non-diapause eggs by injection of anti-diapause hormone rabbit serum into the diapause type of the silkworm, *Bombyx mori*. J Insect Physiol 40: 693–699
Shimizu I, Aoki S, Ichikawa T (1997) Neuroendocrine control of diapause hormone secretion in the silkworm, *Bombyx mori*. J Insect Physiol 43: 1101–1109
Sonobe H, Hiyama Y, Keino H (1977) Changes in the amount of the diapause factor in the suboesophageal ganglion during development of the silkworm, *Bombyx mori*. J Insect Physiol 23: 633–637
Watanabe K (1924) Studies on the voltinism of the silkworm, *Bombyx mori*. Bull Sericult Exp Sta (Tokyo) 6: 411–455
Xu WH, Sato Y, Ikeda M, Yamashita O (1995a) Molecular characterization of the gene encoding he precursor protein of diapause hormone and pheromone biosynthesis activating neuropeptide (DH-PBAN) of the silkworm, *Bombyx mori* and its distribution in some insects. Biochim Biophysic Acta 1261: 83–89
Xu WH, Sato Y, Ikeda M, Yamashita O (1995b) Stage-dependent and temperature-controlled expression of the gene encoding the precursor protein of diapause hormone and pheromone biosynthesis activating neuropeptide in the Silkworm, *Bombyx mori*. J Biol Chem 270: 3804–3808
Yamashita O (1996) Diapause Hormone of the silkworm, *Bomyx mori*: Structure, Gene Expression and Function. J Insect Physiol 42: 669–679
Yamashita O, Hasegawa K (1966) The response of the pupal ovaries of the silkworm to the diapause hormone with special reference to their physiological age. J Insect Physiol 12: 325–330
Yamashita O, Hasegawa K (1970) Oocyte age sensitive to the diapause hormone from the standpoint of glycogen synthesis in the silkworm, *Bombyx mori*. J Insect Physiol 16: 2377–2383
Yamashita O, Hasegawa K (1985) Embryomic diapause. In "Comprehensive Insect Physiology, Biochemistry and Pharmacology Vol 1" Ed by GA Kerkut, LI Gilbert, Pergamon Press, Oxford, pp 407–434

Yamashita O, Irie K (1980) Larval hatching from vitellogenin-deficient eggs developed in male hosts of the silkworm. Nature 283: 385–386

Yu CG, Stay B, Joshi S, Tobe SS (1993) Allatostatin content of brain, corpora allata and haemolymph at different developmental stages of the cockroach, *Diploptera punctata*: Quantification by ELISA and bioassay. J Insect Physiol 39: 111–122

(Received September 17, 2004 / Accepted November 30, 2004)