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Identification of Ecdysis-Triggering Hormone in the Silkworm *Bombyx mori*

Michael E. Adams[1] and Dusan Zitnan
Department of Entomology, University of California, Riverside, California 92521; and Institute of Zoology, Slovak Academy of Sciences, Dubravska cesta 9, 84206 Bratislava, Slovakia

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Ecdysis, the shedding of cuticle at the end of each life stage, is critical to the postembryonic development of insects. The endocrine regulation of ecdysis has been highlighted by the recent description of the epitracheal endocrine system in the tobacco hornworm *Manduca sexta,* which produces ecdysis-triggering hormone (Mas-ETH). This peptide hormone initiates pre-ecdysis and ecdysis through a direct action on the central nervous system. Here we show that ETH-immunoreactivity and ecdysis-triggering activity in epitracheal glands of the silkworm *Bombyx mori* are attributable to a 23 amino acid peptide, Bom-ETH. The complete amino acid sequence of Bom-ETH is SNEAFDEDVMGYVIK-SNKNIPRM-NH_2. Synthetic Bom-ETH was prepared and shown to be chemically and biologically identical to the native substance. Injection of Bom-ETH leads to pre-ecdysis and ecdysis in *B. mori* pharate larvae and pupae as well as comparable stages of *M. sexta*. Exposure of the isolated nervous system to Bom-ETH triggers pre-ecdysis and ecdysis burst patterns corresponding to the natural behavior. Bom-ETH belongs to an extended family of multifunctional neurohormones and hormones found in arthropods and molluscs. © 1997 Academic Press

Growth and development of animals is dependent on the precise coordination of gene expression and endocrinological events. Of particular importance to insect development is the periodic shedding of the exoskeleton known as ecdysis. The endocrine control of ecdysis in *M. sexta* involves the neuropeptide eclosion hormone (1) and ecdysis-triggering hormone (Mas-ETH; 2), which is released by the epitracheal endocrine system. Mas-ETH is a 26-amino acid peptide produced by Inka cells of epitracheal glands, which are segmentally distributed on the ventral surface of tracheal tubes near each spiracle in larvae, pupae and adults.

It seems likely that hormones similar to Mas-ETH trigger ecdysis in all lepidopterans and perhaps even in other insect orders. Such a hypothesis is supported by the occurrence of epitracheal glands in other insect species, including the waxmoth, *Galleria mellonella* (3) and *B. mori* (4). Here we report the isolation and identification of ecdysis-triggering hormone from epitracheal glands of *B. mori*. This peptide is closely related to Mas-ETH with respect to both its structure and function.

MATERIALS AND METHODS

Insects. Eggs of *B. mori* were obtained from Carolina Biological Supply Company (Burlington, NC). Larvae were reared on fresh mulberry leaves on a 16:8 LD photoperiod at 23°C.

Histology. Wholemount nuclear DAPI labelling and immunohistochemical staining with the antisera to FMRFamide and Mas-ETH followed procedures previously described (2, 5). Mas-ETH antiserum was obtained following immunization of rabbits with a 4-branch multiple-antigen peptide, each branch containing the first 18 amino acids of Mas-ETH. Details of the preparation and characterization of this antiserum will be described elsewhere.

Hormone purification and bioassay. Epitracheal glands were dissected from late pharate pupae of *B. mori* and held on dry ice. Crude gland extracts were made by homogenization in Weever's saline, followed by heating at 80°C for 2 min and centrifugation at 10,000g. The supernatant was used for *in vivo* and *in vitro* bioassays. For fractionation by reversed-phase liquid chromatography (RPLC), glands were homogenized in acidic methanol (methanol:water:acetic acid—90:10:1) and centrifuged at 10,000g. The supernatant was partially evaporated and diluted in a mixture of 3% aqueous acetonitrile and 0.1% trifluoroacetic acid prior to RPLC. Initial fractionation of extracts was accomplished with a Microsorb C_4 column (4.5 × 250 mm) using a linear gradient of aqueous acetonitrile in constant 0.1% TFA. Fractions were evaporated to dryness and re-suspended in Weever's saline prior to bioassay. Ecdysis-triggering activity of crude extracts or RPLC fractions was detected by injection into 4th and 5th instar pharate larval silkworms. The active fraction was re-purified using a Vydac C_{18} column under the same conditions as described above.

Amino acid composition, sequencing, mass spectrometry, and chemical synthesis. Edman microsequencing was performed by the Sus-

[1] Corresponding author. Fax: 909-787-3087. E-mail: adams@ucrac1.ucr.edu.

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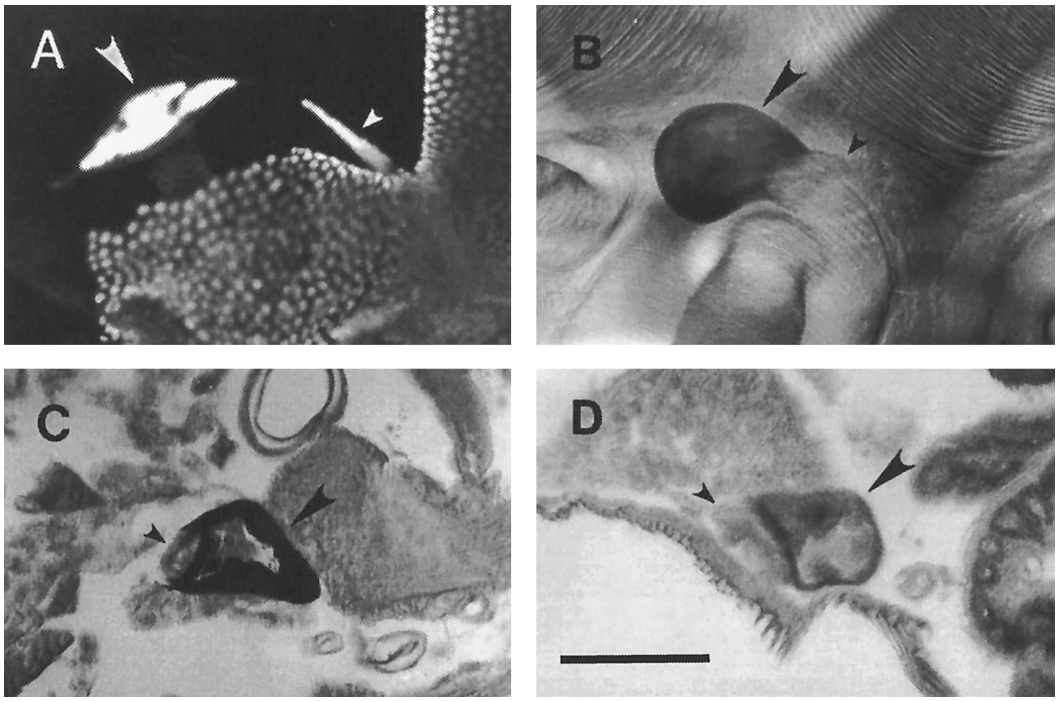

FIG. 1. Histological identification of the epitracheal glands (EG) of pupal stage *Bombyx mori*. In each photomicrograph, the large arrowhead depicts the large, peptidergic Inka cell while the small arrowhead points to smaller associated gland cells. (A) Wholemount nuclear DAPI staining of pharate pupal EG. (B) Wholemount immunohistochemical staining of pharate pupal Inka cell with an antiserum to FMRFamide. (C, D) Reaction of the antiserum to Mas-ETH in Inka cells of pharate pupae at spinning stage (C) and at the onset of ecdysis behavior (D). Notice that Inka cell staining in D is much less intense, indicating prior release of its contents. Scale bar (200 μm) shown in (D) applies to all panels.

sex Centre for Neuroscience (University of Sussex, UK). Electrospray mass spectrometry was carried out at the Analytical Chemistry Instrumentation Facility at the University of California, Riverside. Amino acid composition analysis was performed by the Beckman Center at the California Institute of Technology (Pasadena, CA). Bom-ETH and its corresponding free acid were prepared by Research Genetics, (Birmingham, AL) and kindly provided by the Dupont Corporation.

Electrophysiology. Intact ventral nerve cords of pharate pupal silkworms were dissected in Weever's saline and placed in a 300 μl Sylgard recording chamber. Gland extracts or synthetic Bom-ETH were taken up in Weever's saline and applied to the recording chamber. Extracellular recordings of motor nerve activity were made with polyethylene suction electrodes attached to the dorsal nerve roots of the last 3-4 abdominal ganglia. The neural sheath surrounding the nerve cord was left intact. Signals were amplified with Grass P15 AC amplifiers, taped on a Vetter instrumentation video recorder, and registered on a pen recorder.

RESULTS

Nuclear DAPI staining showed that each epitracheal gland of pharate pupa *B. mori* contains a large Inka cell and two small gland cells (Fig. 1A). Immunohistochemical staining with antisera to FMRFamide and Mas-ETH revealed that the Inka cell is peptidergic (Fig. 1B-D). During pre-ecdysis and ecdysis, Inka cells decrease in size, become transparent, and lose ETH-immunoreactivity (Fig. 1D), suggesting that they release their contents. As previously observed for *M. sexta*, the Inka cells of *B. mori* appear to be involved in the regulation of ecdysis.

Consistent with these observations, crude saline extracts of epitracheal glands showed ecdysis-triggering activity upon injection into pharate larvae and pupae. Injections of 5-25 epitracheal gland equivalents were sufficient to elicit pre-ecdysis and ecdysis. In both stages, pre-ecdysis consists of rhythmic, but asynchronous contractions of different dorsal, lateral and ventral parts of the body wall, which last for about 1 hour. Ecdysis behavior lasts about 10-12 min. and is characterized by reverse peristaltic contractions originating in the most posterior segment and moving anteriorly.

To isolate biologically active components that trigger ecdysis of *B. mori*, epitracheal glands were collected from pharate pupal silkworms and extracted as described in the Materials and Methods section. Gland extracts were fractionated by RPLC (Fig. 2) and bioassayed by injection into pharate larvae. A single active fraction capable of triggering pre-ecdysis and ecdysis was identified (Fig. 2A). An aliquot of this material was subjected to electrospray mass spectrometry, which yielded a molecular mass (MH^+) of 2656.40. The active

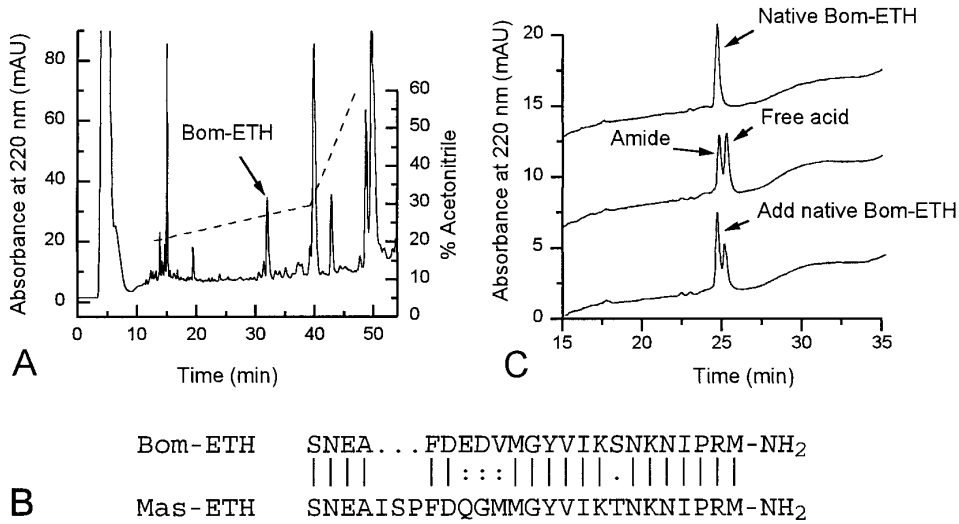

FIG. 2. Fractionation of EG extracts by RPLC and confirmation of structural identity by elution profile. (A) A biologically active peak (Bom-ETH, arrow) elutes at 32 min. This material triggers ecdysis upon injection into pharate larvae and pupae of *B. mori*. (B) Amino acid sequence of Bom-ETH, showing similarity to Mas-ETH. Bom-ETH lacks three amino acids (-ISP-) present in Mas-ETH and shows conservative changes at 4 other positions. (C) Top: Elution profile of native Bom-ETH (100 pmol). Middle: Synthetic Bom-ETH, amide and free -COOH forms (60 pmol each). The -COOH form elutes 30 sec after the amide. Bottom: The same mixture as shown in the middle elution profile has been spiked with 60 pmol of the native peptide. Note that the first peak doubles in size, showing co-elution of the synthetic amide form and the native Bom-ETH.

fraction containing about 75 gland equivalents was subjected to Edman microsequencing, which gave the amino acid sequence shown in Fig. 2B, designated here for the first time as *Bombyx mori* ecdysis-triggering hormone, or "Bom-ETH". Bom-ETH shows 95% sequence similarity to Mas-ETH.

The predicted molecular mass (MH^+) of the amidated form of Bom-ETH is 2656.28, whereas the COOH-terminal form has a predicted molecular mass of 2657.27. Chemical synthesis of both the amide and free-acid form of this peptide was accomplished. These were used as standards for mass spectrometry; MH^+ of the amide form was 2656.5 and that of the free acid was 2657.3. These data support the assignment of the amidated carboxyl-terminus for Bom-ETH. The RPLC profiles and biological activities of the native and synthetic amide were consistent with this assignment. The amidated synthetic Bom-ETH co-eluted with the native material on RPLC, while the free-carboxyl form eluted 30 seconds later (Fig. 2C). Upon injection into pharate larvae, the amidated peptide elicited pre-ecdysis and ecdysis; the free-carboxyl peptide was completely devoid of activity.

Quantitative amino acid composition analysis of synthetic Bom-ETH allowed for an accurate determination of the amount of peptide in a single Inka cell based on peak integrations from RPLC. On this basis, we estimate the amount of Bom-ETH in a single Inka cell to be about 10 pmol in pharate pupae several hours prior to ecdysis.

Injection of Bom-ETH into *M. sexta* pharate larvae and pupae elicited pre-ecdysis and ecdysis. Conversely, injection of Mas-ETH into *B. mori* pharate larvae resulted in the initiation of both pre-ecdysis and ecdysis. Thus, differences in the amino acid sequences between these two peptides are inconsequential for biological activity.

Crude gland extracts (10-25 equivalents) or synthetic Bom-ETH (500 nM - 5 μM) elicited patterned motor bursting from abdominal ganglia of *B. mori* pharate pupae *in vitro* in the absence of the tracheal system (Fig. 3). Both treatments induced within minutes very similar motor patterns; first to appear were short asynchronous bursts, which lasted 45-60 min. These corresponded to the *in vivo* pre-ecdysis behavior. This was followed by regular motor bursts, which appeared first in the most posterior ganglia and passed anteriorly

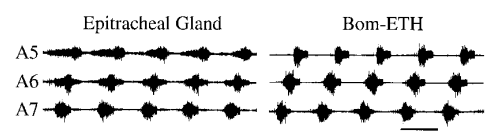

FIG. 3. Ecdysis motor patterns recorded *in vitro* from posterior abdominal ganglia (A5, A6, A7) of the isolated central nervous system of a *B. mori* pharate pupa. (Left) Extract from 25 epitracheal glands elicits ecdysis motor bursts beginning in A7 and moving anteriorly. Delay between each segment is about 3 sec. (Right) Ecdysis bursts elicited from another preparation by addition of 1 nmol to the bath. Calibration mark: 10 sec.

Bom-ETH	SNEA...FDEDVMGYVIKSNKNIPRM-NH_2
Mas-ETH	SNEAISPFDQGMMGYVIKTNKNIPRM-NH_2
Aplysia SCP_B	MNYLAFPRM-NH_2
Mas-CAP 2b	pELYAFPRV-NH_2
Lobster FLI 4	TNRNFLRF-NH_2
Lobster FLI 3	SNRNFLRF-NH_2

FIG. 4. (Top: Bottom) Sequence alignments of *Bombyx* and *Manduca* ETHs with insect, crustacean and molluscan neuropeptides, illustrating the COOH-terminal similarities of this peptide family.

with a delay between segments of about 3 sec. Within each ganglion, burst durations averaged about 8-10 sec and interburst intervals were 8-10 sec. This pattern corresponded closely to the ecdysis behavior observed *in vivo.*

DISCUSSION

We have isolated and identified a new ecdysis-triggering hormone, Bom-ETH, from pharate pupae of *B. mori.* Bom-ETH is a 23 amino acid, carboxyl-terminus amidated peptide which shows high sequence similarity to Mas-ETH previously described in the tobacco hornworm, *M. sexta* (2). The similarity of both chemical structure and function between *B. mori* and *M. sexta* indicates that ecdysis-triggering hormones are conserved in the Lepidoptera. In addition, the sequence of carboxy-terminal amino acids of both Bom-ETH and Mas-ETH show striking similarity to several neuropeptides isolated from the nervous systems of *M. sexta* and other arthropods and molluscs (Fig. 4). These include the cardioactive peptide CAP-2b (6), small cardioactive peptide SCP_B, and lobster FLRF-immunoreactive peptides FLI 3 and FLI 4 (7).

Bom-ETH is 95% similar to Mas-ETH; the major difference is the deletion of three internal amino acids -Ile-Ser-Pro- present in Mas-ETH. The loss of proline in Bom-ETH is expected to eliminate a bend presumably present in Mas-ETH. Other differences in the two peptide sequences are relatively minor. Both peptides have identical N- and C-termini, and both are amidated at the carboxy-terminus. We have shown in this work that C-terminal amidation of Bom-ETH is essential for biological activity. Although this was not demonstrated in our earlier account of Mas-ETH, our data suggest that the same will hold for this peptide.

Bom-ETH appears only to be produced and released from the large peptidergic Inka cells of each epitracheal gland. This is confirmed by the appearance of ETH immunoreactivity and electron dense endocrine granules in the Inka cell before each ecdysis and loss of ETH staining and granules at the onset or after ecdysis (2, 4). ETH immunoreactivity or presence of peptidergic granules were never observed in the smaller gland cells which also comprise part of the epitracheal gland (3, 4).

Extracts of epitracheal glands trigger pre-ecdysis and ecdysis in isolated ventral nerve cords dissected from pharate pupae. We have shown that 15-25 cell equivalents are sufficient to trigger these behaviors *in vitro.* The observation that the frequency and duration of ecdysis bursts *in vitro* match the parameters of muscle contractions *in vivo* confirms that ecdysis behavior is completely driven by oscillatory circuitry intrinsic to the central nervous system, and that sensory input is not required to determine the characteristics of the behavior.

Injection of Bom-ETH induces within minutes pre-ecdysis followed by ecdysis of pharate larvae and pupae. Our estimate that each pharate pupal Inka cell contains 10 pmol of Bom-ETH leads to the inference that the peptide probably reaches several hundred nanomolar concentration during the pre-ecdysis and ecdysis sequence. The physiologically effective levels of Bom-ETH *in vitro* are in agreement with this inference and are also comparable to those previously observed for Mas-ETH in *M. sexta* (2).

The identification of Bom-ETH provides tangible evidence that the epitracheal endocrine system and its associated peptide hormones are involved in triggering ecdysis and possibly other associated events in different Lepidopteran families and possibly other insect orders as well. The phylogenetic range of the epitracheal endocrine system and its homologous organs, as well as conservation of the structure and function of ecdysis triggering hormones remain to be determined. Bom-ETH is the newest member of a family of C-terminally-amidated peptides that include FMRF-amide-related peptides (7, 8) and SCPs (9-11).

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