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Molecular cloning and structural characterization of Ecdysis Triggering Hormone from *Choristoneura fumiferana*

Bhagath Kumar P.[a], Kasi Viswanath K.[a], Tuleshwori Devi S.[a], Sampath Kumar R.[a], Daniel Doucet[b], Arthur Retnakaran[b], Peter J. Krell[c], Qili Feng[b], Dinakara Rao Ampasala[a,*]

[a] *Centre for Bioinformatics, School of Life Sciences, Pondicherry University, India*
[b] *Natural Resources Canada, Sault Ste. Marie, Ontario, Canada*
[c] *Department of Microbiology and Cellular Biology, University of Guelph, Guelph, Ontario, Canada*

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ABSTRACT

At the end of each stadium, insects undergo a precisely orchestrated process known as ecdysis which results in the replacement of the old cuticle with a new one. This physiological event is necessary to accommodate growth in arthropods since they have a rigid chitinous exoskeleton. Ecdysis is initiated by the direct action of Ecdysis Triggering Hormones on the central nervous system. *Choristoneura fumiferana* is a major defoliator of coniferous forests in Eastern North America. It is assumed that, studies on the ecdysis behavior of this pest might lead to the development of novel pest management strategies. Hence in this study, the cDNA of *Cf*ETH was cloned. The open reading frame of the cDNA sequence was found to encode three putative peptides viz., Pre-Ecdysis Triggering Hormone (PETH), Ecdysis Triggering Hormone (ETH), and Ecdysis Triggering Hormone Associated Peptide (ETH-AP). The *Cf*ETH transcript was detected in the epidermal tissue of larval and pupal stages, but not in eggs and adults. In order to explore the structural conformation of ETH, ab initio modelling and Molecular Dynamics (MD) Simulations were performed. Further, a library of insecticides was generated and virtual screening was performed to identify the compounds displaying high binding capacity to ETH.

1. Introduction

The growth and development of insects is characterized by periodic events of synthesis and degradation of the exoskeleton between successive instars; the complete process is known as molting, which ends with shedding of the old exoskeleton called ecdysis. Many studies have reported the key role of various hormones in molting and metamorphosis in insects [1–4]. The molting process is regulated by a precisely-timed gene expression program orchestrated by the steroid hormone 20-hydroxyecdysone (20E), secreted at the end of each instar [5]. Both the rise and fall of 20E levels are critical for molting. Rising 20E titres trigger apolysis, by which the old cuticle is separated from the epidermis, and is accompanied by the dissolution of the old cuticle and synthesis of new cuticle. Falling 20E titres initiate a sequence of events which are required for the actual shedding of the exoskeleton [6,7].

A collection of peptide hormones and neurotransmitters act in concert along with 20E to ensure the proper unfolding of each ecdysis phase [8]. Among them, ETHs are blood-borne peptides that act directly on the central nervous system (CNS) to coordinate the skeletal muscle contractions occurring at the pre-ecdysis and ecdysis phases [9]. Most insects produce two different ETHs, called PETH and ETH in Lepidopterans, and ETH1 and ETH2 in other insects. Both ETHs are cleaved from a unique precursor peptide produced and secreted by specialized secretory cells, called Inka cells located in the epitracheal glands [10]. Eclosion hormone (EH) promotes the release of Inka cell peptides into the haemolymph [11]. ETH and related peptides were identified from the epitracheal glands of *Manduca sexta*, *Bombyx mori* and *Drosophila melanogaster* [6,12–14].

Null mutations in the *Drosophila* ETH gene show severe defects that include failure in respiratory system inflation, and are incapable to perform ecdysis behavioural sequence, leading to lethality during first ecdysis which suggests that ETH is crucial for insect ecdysis. However, normal ecdysis was observed in mutant larvae when synthetic ETH was injected [15]. Circulating ETHs bind to a receptor (ETHR) expressed by specific neurons of the CNS. Discovery of ETHR in *Drosophila* [16,17] and *Manduca* [18] facilitated the

* Corresponding author at: Centre for Bioinformatics, School of Life Sciences, Pondicherry University, Kalapet, Puducherry-605014, India.
E-mail address: ampasaladr@bicpu.edu.in (D.R. Ampasala).

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functional analysis of ETH-mediated control of ecdysis by the CNS. The ETHR gene encodes two G protein-coupled receptor subtypes (ETHR-A and ETHR-B) generated via alternative splicing. ETHR-A and ETHR-B are expressed mostly in non-overlapping sets of central neurons. ETHR expression corresponds with the attainment of CNS sensitivity to PETH and ETH, and its ability to direct the ecdysis sequence [18,19]. ETHs and ETHRs have been extensively characterized from the moths *M. sexta*, *B. mori*, *D. melanogaster*, *Tribolium castaneum* and *Aedes aegypti* [6,15,17,19–24].

Dai and Adams (2009) presumed the possibility of ETH as a target to regulate the ecdysis behavioural sequence in *A. aegypti*. Release of ETH is generally observed on or before every molt, only appearing for an extremely brief period of time, triggering ecdysis. After this sudden rise and decline in concentrations, it has no other putative function. Though blocking ETH action would be lethal for the insect; using insecticides with shorter half-life will not help in effectively eradicating the pest. Furthermore, inappropriate use of chemicals at higher rate and at improper life stage of the pest confers resistance in a few species and eliminate others, creating imbalances in the ecosystem. Thus the close monitoring of pest's life cycle helps in calculating the time required for a pest to grow and proper time for application of pesticides, as pest population infesting a particular crop or an area generally grows synchronously. This requires a complete study of the life cycle of the pest species and molecular expression profiles of targeted proteins, which is a very important aspect of integrated pest management. Thus, cloning ETH mRNA and studying its expression profile is necessary as it helps in understanding the structural features of ETH and also provides information regarding the mechanism of binding to its receptor.

Knowledge about the three-dimensional (3D) structure of ETH and information regarding its mechanism of action is lacking which would otherwise assist in designing potential inhibitors and hinder its function in molting. But isolation and crystallization of proteins is challenging and could be a limiting factor in structure determination. Computational methods have notable success in modelling protein structures with sufficient accuracy to facilitate functional annotation, biochemical analyses, and biological characterization [25]. Though homology modelling is the most reliable method, it is not applicable to proteins lacking a template with significant sequence similarity [26]. In such a case the alternative methods include, de novo or ab initio modelling, which predict the structure from sequence alone, without relying on other known structures [27]. These approaches were also widely used and proved to be successful computational methods to model the 3D structure of a protein accurately [28–30]. Although these methods require huge computational resources, they help us in understanding the principles behind protein folding and also provide potential benefits in fields like medicine and drug design [31].

Hence in this study, we performed the molecular cloning of the cDNA encoding PETH, ETH, and ETH-AP peptides from the spruce budworm, *Choristoneura fumiferana*, and investigated its developmental and tissue-specific expression. We further used ab initio method to predict the 3D structure of ETH and performed molecular docking studies to identify potential ETH-binding molecules using High Throughput Virtual Screening approach (HTVS). These experiments could provide basis for the development of compounds that could block the ecdysis process in this important pest species.

2. Materials and methods

2.1. Insect culture

Eastern spruce budworm (*C. fumiferana* Clem., Lepidoptera: Tortricidae) eggs were maintained at 22 °C and 70% relative humidity (RH) and allowed to hatch into first instar larvae on balsam fir (*Abies balsamea*) needles. Larvae took six days to molt into second instar which were further allowed to grow at 16 °C for one week and then stored for 27 weeks at 2 °C to complete their diapause. Following diapause, larvae were transferred to 16 °C for one week and fed with artificial diet [32], maintained at 22 °C, 70% RH, with a photoperiod of 12-h light and 12-h darkness until they reached the adult stage.

2.2. RNA isolation

Total RNA from *C. fumiferana* was isolated using the guanidium thiocyanate method [33]. Samples included whole eggs, larvae, pupae, and adult moths, as well as epidermal, fat body, and midgut tissues dissected from 6th instar larvae. For cDNA library construction (see Section 2.3), total RNA was isolated from larvae collected at various time intervals during the 5th–6th instar molt. All samples were stored at −80 °C until ready for cDNA library construction and northern blotting.

2.3. Cloning of CfETH cDNA

An expressed sequence tag (EST) library was generated from RNA collected during the 5th–6th instar molt (Q. Feng, unpublished data). Briefly, the library was constructed using the Uni-ZAP XR vector (Agilent technologies, Mississauga, Ontario) according to the manufacturer's protocol. Following mass in vivo excision and plating of the library on LB/ampicillin media, 5280 colonies were picked and the pBluescript vector insert was amplified using T7 and T3 primers. 4073 PCR products were successfully generated, of which 2947 reactions yielded sequence with a phred Q-score > 20. All sequences are accessible via the NCBI accession numbers FE271138.1–FE274084.1. ESTs encoding putative *Cf*ETH sequences were identified by performing translated BLAST (tBLASTn) using ETH sequences from *Bombyx* and *Manduca* as queries. The full length *Cf*ETH cDNA sequence was obtained by completely sequencing the EST clone (the best BLAST match) in both directions. Finally the obtained sequence was deposited in GenBank (GeneBank ID: JX878448.1) using sequin software.

2.4. Northern blot analysis

Northern blotting was performed as described in Ampasala et al. 2004. Electrophoresis was performed by loading 10 μg of total RNA per lane on 1.2% formaldehyde agarose gel. Resolved RNAs were transferred onto nylon membranes, positively charged (Hybond-XL, Amersham) by vacuum blotting for 2–3 h. Nucleic acid cross-linking was done in an UVcross linker (Stratagene Cloning Systems, La Jolla, CA, USA) and membranes were incubated at 65 °C for three hours in a pre-hybridization solution (Rapid-hyb buffer, Amersham Pharmacia Biotech). A 400 bp fragment of the *Cf*ETH cDNA was labeled with ^{32}P-dCTP and used as a hybridization probe. Membranes were then hybridized at 65 °C for at least 12 h in a hybridization solution of Rapid-hyb buffer containing the *Cf*ETH probe (2×10^6 cpm/ml). Following hybridization, membranes were washed twice in 2X SSC plus 0.1% SDS at 42 °C for 15 min and twice with 0.5X SSC plus 0.1% SDS at 55 °C for 15 min and once with 0.1X SSC plus 0.1% SDS at 65 °C for 15 min. Membranes were then exposed to X-ray film in a Kodak cassette with intensifying screen at −75 °C. The RNA size was determined by running a RNA ladder (0.24–9.5 kb RNA Ladder, Invitrogen life technologies) on the same gel.

2.5. Multiple sequence alignments and phylogenetic analysis

The insect ETH protein sequences used in this study were listed in Table 1. Multiple sequence alignments were performed using