Structure-Function Analysis of Ecdysis Triggering Hormone (ETH) in *Manduca sexta*

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Abstract

is is a stereotyped behavior that is regulated by several neuropeptides and results in
l shedding of the old cuticle. We have been examining the structure-function
f one of the main neuropeptides regulating ecdysis in the tobacco hornworm,
a, namely Ecdysis Triggering Hormone (ETH). ETH is a 26 amino acid peptide
the CNS to initiate ecdysis behaviors. Activation of the cell 27/704 group in the
cGMP-mediated mechanism, leads to the onset of the ecdysis motor program.
l analogs were assayed for their ability to induce biological activity via 1) induction
haviors *in vivo*, 2) the activation of cGMP within the cell 27/704 group *in vitro*, as
unohisto-chemically, and 3) activation of the ecdysis motor program, as measured
logically. Non-amidated ETH was not able to elicit ecdysis behaviors *in vivo*, nor
rons show characteristic increases in cGMP staining. This suggests that amidation
for biological activity. N-terminal-truncated versions of amidated ETH elicited
nses. Analogs shorter than 12 amino acids were not able to initiate ecdysis
ivo at physiological concentrations, nor did the neurons stain for cGMP. However,
cal concentrations of shorter fragments did elicit responses *in vivo*, and
doses acted *in vitro* to activate the cell 27/704 group. Thus, our results suggest that
y-terminal moieties of ETH are sufficient to confer biological activity in *M. sexta*,
ation is necessary for this activity.

Introduction

vth of the larval body is accommodated by successive molts of the old cuticle,
v and larger cuticle in its place. This is the process of ecdysis, a highly conserved
ughout arthropods, which is under tight neuro-endocrine control. Ecdysis can be
distinct categories; "pre-ecdysis", or the loosening of the cuticle, and "ecdysis", or
he cuticle. These behaviors can be observed *in vivo* as contractions of the body
ysis is characterized by synchronous contractions, and ecdysis is characterized by
ves that contract posterior to anterior along the length of the animal. In *vitro*,
changes associated with ecdysis include increases in cGMP in neurons triggering
or programs, and changes in rhythmicity of bursting of the motor neurons
These changes can be monitored by immunohistochemistry (Ewer *et al.*, 1994) and
logy (Weeks & Truman, 1984; Gammie & Truman, 1997), respectively. Both of
ors are controlled by neuropeptides: Pre-Ecdysis Triggering Hormone (**PETH**)
osening of the cuticle, and Ecdysis Triggering Hormone (**ETH**) controls both
d shedding of the cuticle. Shedding is triggered by activation of homologous
e SEG, thorax and abdomen, via a cGMP mediated mechanism (**Fig. 1**).
l is a 26 amino acid peptide (Zitnan *et al.*, 1996), and shares a degree of sequence
th PETH (a 13 amino acid peptide; **Fig. 2**), but both cause different behaviors
s. We are interested in the structure/function relationship of ETH. A search for
e moieties within ETH can be attained through truncations of synthetic peptides,
vidual effects on animals *in vivo*, and on cells regulating ecdysis behaviors *in vitro*.

1. Design Truncations of ETH

mpare PETH:	SF	IKPN -NVPRV-NH$_2$
ETH:	SNEAISPFDQGMMGYV	IKTNKNIPRM-NH$_2$
21 mer:	SPFDQGMMGYV	IKTNKNIPRM-NH$_2$
12 mer:	YV	IKTNKNIPRM-NH$_2$
11 mer:	V	IKTNKNIPRM-NH$_2$
10 mer:		IKTNKNIPRM-NH$_2$
7 mer:		NKNIPRM-NH$_2$
6 mer:		KNIPRM-NH$_2$
5 mer:		NIPRM-NH$_2$

ETH truncations from the C-terminus, conserved residues shown in red

Goal

mine the biologically active moieties of ETH in *in vitro*

Materials and Methods

4th stage larvae were monitored for signs of air-filling in the old slipped head capsule, as an index of when they would begin ecdysis. These animals were injected with approximately 100 pmol of synthetic ETH (or analogs) 1-2 hours later,or the CNS was dissected and incubated with ETH. This method induces a very consistent timing of onset of ecdysis (Ewer *et al.*, 1994).

1)ETH Peptide analogs

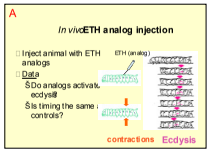
Analogs truncated from the N-terminus, as well as non-amidated ETH were synthesized (Peptron, Inc) and tested at equal concentrations on *in vivo* and *in vitro* bioassays (see Fig. 1).

2) In vivo analysis

Ecdysis behaviors were monitored and timing of onset of the behaviors was recorded to the minute (Fig. 2a).

3) In vitro analysis

CNS was assessed for increases in cGMP using cGMP-specific antibodies. Tissues were scored blindly for intensity of stain in subesophageal, thoracic and abdominal ganglia. Induction of the pre-ecdysis and ecdysis motor programs were monitored electrophysiologically with dual recording electrodes at consecutive ganglia (Fig. 2b).



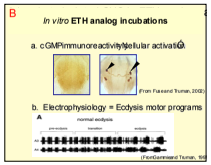


Fig. 2 A *in vivo* injection approach Showing peristaltic waves (contractions shown in arrows)
Fig. 2 B *in vitro* approach; cGMP stains and electrophysiological recordings from 26 mer.

Results

Amino Acid Order, Red denotes similarity	Analog	Biological Activity (min ± sem) [100 pmol]	[100 nmol]
SFIKPN NVPRV NH$_2$	PETH		
SCEAISPFDQGMMGYVIKTNKNIPRM-NH$_2$	26 mer	42 ± 1 (59/60)	
SPFDQGMMGYVIKTNKNIPRM-NH$_2$	21 mer	40 ± 3 (19/19)	
YVIKTNKNIPRM-NH$_2$	12 mer	42 ± 2 (22/22)	
VIKTNKNIPRM-NH$_2$	11 mer	(0/14)	(3/3)
IKTNKNIPRM-NH$_2$	10 mer	(0/16)	(3/3)
NKNIPRM-NH$_2$	7 mer	(0/5)	(4/7)
KNIPRM-NH$_2$	6 mer	(1/5)	(3/4)
NIPRM-NH$_2$	5 mer	(0/6)	(0/3)

Fig. 3 *in vivo* table; sequence homology denoted in red. (ratio of animals undergoing Ecdysis / unable to complete Ecdysis.

Analog	SEG stain	TG Stain	Abdominal stain
26mer	9/9 1/9	9/9	8/9 1/9
12mer	6/10 3/10	9/11 1/11	6/11 3/11
7mer	3/3	3/3	1/3 2/3
6mer	0/4 2/4	0/4 2/4	0/4 0/4

Fig. 4 cGMP staining chart, black = strong stain, grey = weak stain.

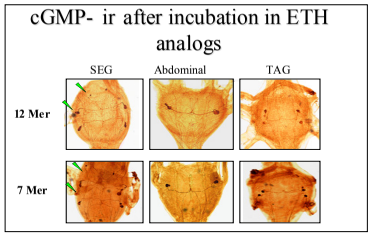


Fig. 5 Representative Staining pictures from assorted ganglia.

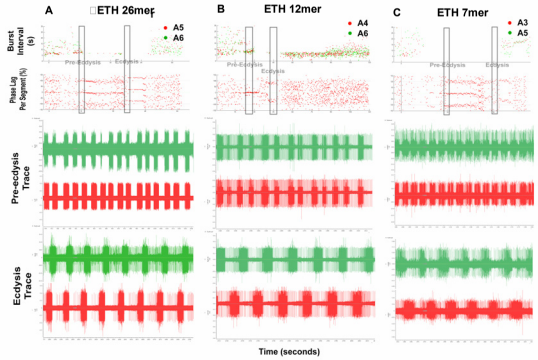


Fig. 7 Bursts were detected from spike trains band-pass filtered with Gaussian kernels, which generate a smooth local measure of firing rate. Bursts were identified by local minima, and complex burst shapes distinguished by aggregating bursts to minimize the local variance in the serial differences in maximum burst height. This analysis reveals the burst period at each recording site (defined as the distance between median spike for successive bursts) and the phase delay

Electrophysiology Results

Analog [[10^-6]	Pre-Ecdysis	Ecdysis
26 mer	(9/11)	(8/11)
12 mer	(2/2)	(2/2)
11 mer	(2/2)	(1/2)
10 mer	(2/2)	(1/2)
7 mer	(6/7)	(6/7)
6 mer	(2/3)	(0/3)

Summary

Size	Activity (*In vivo*)	cGMP (*In vitro*)	Electrophysiolo (*motor program*)
26 Mer	Y	Y	(8/11)
12 Mer	Y	Y	(2/2)
11 Mer	Y [high]		(1/2)
7 Mer	Y [high]	(Y)	(1/2)
6 Mer	(Y) [high]		(6/7)
5 Mer	N [high]	N	(0/3)

Fig. 8 Summary table: *in vivo* ecdysis behaviors; black [100pmol], grey [100nmol] *In vitro* Y=staining, (Y)=light stain. Electrophysiological neuronal activation represents animals that undergo both Pre-Ecdysis and Ecdysis.

Discussion

Conservation of function appears to exist at the C-terminus, since no
26 amino acid peptide (26mer) does not induce biological activity *in vivo*
(electrophysiologically), nor does it induce increases in cGMP *in vitro*.

The minimal amidated moiety to confer biological activity appears t
which induces strong pre-ecdysis and modified ecdysis motor patterns
increases in cGMP in a fraction of preparations monitored. ETH has l
to act downstream of the target neurons in *Drosophila* (Park *et*
suggesting that this may be the case in preparations where cGMP immu
was not noted.

Degradation of truncated ETH by hemolymph peptidases may acc
fact that only pharmacological concentrations of 7mer induced ecdysis b
vivo. This appears to be at the 12 amino acid residue, since addition
moieties to bioinactive 10mer and 11mer restores biological
physiological concentrations. This still needs to be verified.

Delays in activation by the small truncated peptides may be
presence of two receptors for ETH. Electrophysiological recordi
ecdysis motor patterns show significant delays in activation compared
while pre-ecdysis behaviors appear normal. This suggests differe
moieties on pre-ecdysis and ecdysis receptors by 7mer peptides. Th
putative ETH receptors in *Drosophila* (Park et al., 2003) and multipl
pathways have been shown to exist (Clark *et al.*, 2004).

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