

# Ecdysteroidogenesis in *Heliothis virescens* (Lepidoptera: Noctuidae): Recombinant Prothoracicotropic Hormone and Brain Extract Show Comparable Effects

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## Abstract

Prothoracicotropic hormone (PTTH) is a neuropeptide that triggers a cascade of events within the prothoracic gland (PG) cells, leading to the activation of all the crucial enzymes involved in ecdysone biosynthesis, the main insect steroid hormone. Studies concerning ecdysteroidogenesis predicted PTTH action using brain extract (BE), consisting in a complex mixture in which some components positively or negatively interfere with PTTH-stimulated ecdysteroidogenesis. Consequently, the integration of these opposing factors in steroidogenic tissues leads to a complex secretory pattern. A recombinant form of prothoracicotropic hormone (rPTTH) from the tobacco budworm *Heliothis virescens* (F) (Lepidoptera: Noctuidae) was expressed and purified to perform in vitro tests in a standard and repeatable manner. A characterization of rPTTH primary and secondary structures was performed. The ability of rPTTH and *H. virescens* BE to stimulate ecdysteroidogenesis was investigated on the third day of fifth larval stage. rPTTH activity was compared with the BE mixture by enzyme immunoassay and western blot, revealing that they equally stimulate the production of significant amount of ecdysone, through a transduction cascade that includes the TOR pathway, by the phosphorylation of 4E binding protein (4E-BP) and S6 kinase (S6K), the main targets of TOR protein. The results of these experiments suggest the importance of obtaining a functional pure hormone to perform further studies, not depending on the crude brain extract, composed by different elements and susceptible to different uncontrollable variables.

**Key words:** ecdysteroidogenesis, prothoracic glands, recombinant prothoracicotropic hormone, brain extract, *Heliothis virescens*

In insects, ecdysteroids biosynthesis and secretion by prothoracic glands (PGs) or analogous organs play an essential role in molting and metamorphosis regulation (Iga and Kataoka 2012, Hentze et al. 2013). Following the neuropeptidic stimuli of prothoracicotropic hormone (PTTH), PGs secrete ecdysone (McBrayer et al. 2007, Iga and Kataoka 2012). PTTH is produced by the neurosecretory cells of the brain, the *pars intercerebralis*, and it is stored in the *corpora cardiaca*, a pair of neuroglandular organs located behind the brain (Agui et al. 1979, Dai et al. 1994, Gullan and Cranston 2005). Once released by *corpora cardiaca*, PTTH acts on PGs, binding the specific tyrosine kinase receptor, Torso, on the cellular membrane, in order to activate multiple signal transduction cascades (Rewitz et al. 2009). To date, the entire process involving PTTH stimulation and protein biosynthesis in

PGs is not completely elucidated, but it is clear that PTTH positively modulates cAMP increase and intracellular Ca<sup>2+</sup> release, as observed in *Manduca sexta* (Linnaeus, 1763) (Lepidoptera: Sphingidae) (Smith et al. 1985, 1986; Fellner et al. 2005) and *Bombyx mori* (Linnaeus, 1758) (Lepidoptera: Bombycidae) (Gu et al. 1996, 1998). Moreover, following the binding with its specific receptor, PTTH leads to the activation of two specific signal transduction cascades: MAPK and PI3K/Akt/TOR. These two pathways, differently activated depending on lepidopteran species, promote protein translation of various enzymes resulting in ecdysone biosynthesis from cholesterol. It was demonstrated that in *B. mori* (Lin and Gu 2007, Gu et al. 2012) and *Heliothis virescens* (Fabricius, 1777) (Scieuzo et al. 2018) both pathways are involved in PTTH-stimulated ecdysteroidogenesis, whereas in *M. sexta*,

the only one MAPK pathway seems to be activated (Rybczynski et al. 2001). In *M. sexta*, *B. mori*, and *H. virescens*, PTTH stimulation also increases protein biosynthesis and phosphorylation, in order to support ecdysone production by PGs (Rybczynski and Gilbert 1994, Pennacchio et al. 1997, Gu et al. 2012). PTTH was firstly isolated and characterized in *B. mori* (Kataoka et al. 1991), in which it is synthesized as pre-pro-hormone and subsequently processed in the brain, to obtain a glycosylated homodimer with active subunits of 109 amino acids, with three intra- and one interchain disulfide bonds (Ishibashi et al. 1994). *Heliothis virescens* PTTH shows high similarity with the entire *Helicoverpa zea* (Boddie, 1850) (Lepidoptera: Noctuidae) PTTH sequence (Wei et al. 2005) and with the active portion of *B. mori*, *M. sexta*, *Antheraea pernyi* (Guerin Meneville, 1855) (Lepidoptera: Saturniidae), *Samia cynthia ricini* (Drury, 1773) (Lepidoptera: Saturniidae), and *Hyalophora cecropia* (Linnaeus, 1758) (Lepidoptera: Saturniidae) PTTH sequence (Xu et al. 2003). In this work, a recombinant form of *H. virescens* PTTH (rPTTH), suitable to perform in vitro tests in a standard and repeatable manner, was expressed and purified. Moreover, the functionality of increasing concentration of rPTTH on PGs was evaluated by in vitro tests (enzyme immunoassay and western blot), in comparison to *H. virescens* PTTH contained in the brain extract (BE). This is to define whether the rPTTH alone or other components of the BE are responsible for triggering the transduction cascade and modulate ecdysteroidogenesis in PGs.

Results highlight that PTTH is the active component of the BE mixture, strengthening the results obtained in previous studies performed using the BE and aimed to clarify the mechanism by which the hormone stimulates ecdysteroidogenesis.

## Materials and Methods

### Insect Rearing and Staging

*Heliothis virescens* larvae were reared according to Vinson et al. (1973) on a standard artificial diet developed by Vanderzant et al. (1962). Rearing temperature was maintained at  $29 \pm 1^\circ\text{C}$ , under a photoperiod of 16:8 (L:D) and relative humidity  $70 \pm 5\%$ . Last (fifth) instar larvae were staged according to Webb and Dahlman (1985) and synchronized as reported by Pennacchio et al. (1992).

### RNA Extraction, cDNA Synthesis, and Polymerase Chain Reaction

Total RNA from an equal number of brains collected from 3- to 4-d-old last (fifth) instar larvae was extracted using TRI-Reagent (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's protocol. An additional DNase treatment (Invitrogen, Waltham, MA) was carried out before the second purification step to remove any contaminating DNA. RNA was further purified by the RNeasy MinElute Clean-up Kit (Qiagen, Venio, the Netherlands) and eluted in 20  $\mu\text{l}$  of RNA Storage Solution (Ambion Inc., Austin, TX), following the manufacturer's protocol. RNA integrity was verified on an Agilent 2100 Bioanalyzer using RNA nano chips (Agilent Technologies, Palo Alto, CA) and RNA quantity was determined by a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). cDNA coding for the mature active form of PTTH was obtained using 5  $\mu\text{g}$  of total RNA by SuperScript III Reverse Transcriptase (Invitrogen), according to the manufacturer's protocol. cDNA was amplified by polymerase chain reaction (PCR), using the following primers:

*Hvir*PTTHpET32FOR: 5'-GACGACGACAAGATGGGTAACATTAAGTTCGAGAAG-3'  
*Hvir*PTTHpET32REV: 5'-GAGGAGAAGCCCGGTTATTTCTCAGTAGCGTAGTAA-3'

Primers were designed starting from the nucleotide sequence of PTTH cDNA (AY172671.1), obtained from the databases available at the National Center for Information and Biotechnology (NCBI) ([www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)). The underlined extensions were added to allow a direct cloning of the fragment into the expression vector pET32 Ek/LIC, in frame with Trx, His, and S tags (a total of 17 kDa). PCR amplification was performed using the high-fidelity Taq, KOD DNA Polymerase (Novagen, Madison, WI), according to the manufacturer's protocol, in a thermocycler GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA).

### Cloning, Expression, and Purification of rPTTH

The amplimer, coding for the active form of PTTH, was purified using Quantum Prep Freeze N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad, Hercules, CA) and ligated into pET-32 Ek/LIC vector (Novagen) for bacterial expression, according to the manufacturer's instructions (Ek/LIC Cloning Kits, Novagen). The construct was sequenced (Macrogen Europe, the Netherlands) and transformed into *E. coli* BL21 (DE3) chemically competent cells (Novagen) according to the manufacturer's protocol. The recombinant mature form of PTTH was expressed growing the transformed *E. coli* BL21 (DE3) cells at  $37^\circ\text{C}$  in Luria Bertani broth (LB). rPTTH gene expression was induced at  $\text{OD}_{600} = 0.6$  by adding 0.5-mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). After growing for additional 4 h at  $37^\circ\text{C}$ , rPTTH was purified under denaturing conditions using affinity chromatography on nickel resin Qiagen Ni-NTA (Qiagen Ni-TED packed columns 1000-Macherey-Nagel, Bethlehem, PA) following the manufacturer's protocol. The purified protein was dialyzed against phosphate-buffered saline (PBS) 1 $\times$ , to remove urea and allow the rPTTH refolding in an homodimeric form, essential for its biological activity (Ishibashi et al. 1994). A rate of dialyzed peptide was used to verify the correct folding by native PAGE (12.5%). Proteins were prepared in a nonreducing and nondenaturing sample buffer (neither SDS in electrophoresis gel and buffer components nor  $\beta$ -mercaptoethanol in sample loading buffer) to preserve both the secondary structure and the native charge density of the protein. The purified fusion protein was then digested by 0.01 units of thrombin/ $\mu\text{g}$  of purified protein (thrombin from bovine plasma 1,000 IU/mg, Sigma-Aldrich) for 16–18 h at room temperature ( $25^\circ\text{C}$ ) in order to remove the Trx and the His tags. Thrombin was finally inactivated by heating the sample at  $60^\circ\text{C}$  for 10 min. The sample was analyzed by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) (15%) in order to verify the occurred digestion. Digested rPTTH was then separated from His-tag, using affinity chromatography on nickel resin Qiagen Ni-NTA (Qiagen Ni-TED packed columns 1000-Macherey-Nagel) and imidazole gradient, following the manufacturer's protocol. Then, in order to remove imidazole, dialysis against PBS 1 $\times$  was performed for 16 h. A rate of dialyzed peptide was used to verify the integrity by SDS-PAGE (15%) in the presence and in the absence of the reducing agent  $\beta$ -mercaptoethanol (Kataoka et al. 1991, Ishibashi et al. 1994, Shionoya et al. 2003).

### Structural Characterization of rPTTH

rPTTH was treated with 60  $\mu\text{l}$  of 10-mM DTT in 50-mM  $\text{NH}_4\text{HCO}_3$ , pH 8.5 for 45 min at  $56^\circ\text{C}$  to reduce cysteine residues and then with 55-mM iodoacetamide in the dark at room temperature for 30 min for the alkylation reaction. The protein was finally digested with trypsin (1/50 w/w) in 50 mM  $\text{NH}_4\text{HCO}_3$ , pH 8.5 overnight at  $37^\circ\text{C}$ . The resulting peptide mixture was directly analyzed by both MALDI tandem mass spectrometry (MALDI-MS/MS) on a MALDI

TOF-TOF 5800 plus instrument (ABI Sciex, Framingham, MA) and liquid chromatography/tandem mass spectrometry (LC-MS/MS) using an LTQ Orbitrap XL Orbitrap mass spectrometer (Thermo Fisher Scientific). CD spectra were recorded at 25°C using 1-cm quartz cells in the range between 260 and 198 nm using a Jasco J-1100 CD spectrometer (Jasco, Easton, MD). Protein concentration for CD measurement was 5  $\mu$ M in 10-mM sodium phosphate buffer, pH 7.4.

### Extraction of PTTH

The brain extract mixture containing PTTH (hereafter referred as BE) was prepared by homogenizing brains dissected from an equal number of *H. virescens* 3-d (V3) and early 4-d (V4) last (fifth) instar larvae and stored in ice-cold Grace's insect medium (Sigma-Aldrich). The homogenate was placed in boiling water for 2 min, cooled to 4°C on ice and centrifuged at 15,000 g at 4°C for 5 min (Pennacchio et al. 1997). Before being used, BE was diluted in Grace's insect medium to 0.1 brain equivalent/ $\mu$ l and used immediately for the experiments described below or stored at -80°C.

### In Vitro Ecdysteroidogenesis Stimulation

For dose-response experiments, PGs from 3-d (V3) old last (fifth) instar larvae were dissected at room temperature, in PBS 1 $\times$  as previously reported (Pennacchio et al. 1998). Individual glands were held in cell culture wells in 100  $\mu$ l of Grace's insect medium (Sigma-Aldrich) for 30 min (time of rest) at 25°C, in order to reduce the possibility of their activation by experimental manipulation, as reported for *Manduca sexta* PGs (Bollenbacher et al. 1983, Smith et al. 1986). Following the dissection of PGs, Grace's insect medium was replaced with a fresh medium containing BE or rPTTH. In vitro ecdysone biosynthesis was evaluated for a single PG incubated under different conditions: control PG incubated in Grace's insect medium (Sigma-Aldrich); stimulated PG with 0.1 brain equivalent/ $\mu$ l BE; stimulated PG with 0.1 ng/ $\mu$ l rPTTH; stimulated PG with 0.25 ng/ $\mu$ l rPTTH; stimulated PG with 0.5 ng/ $\mu$ l rPTTH. Each condition was maintained for 3 h at 25°C. The production of ecdysone after in vitro stimulation was determined by a competitive enzyme immunoassay (EIA), as previously described (Scieuzo et al. 2018). All experiments

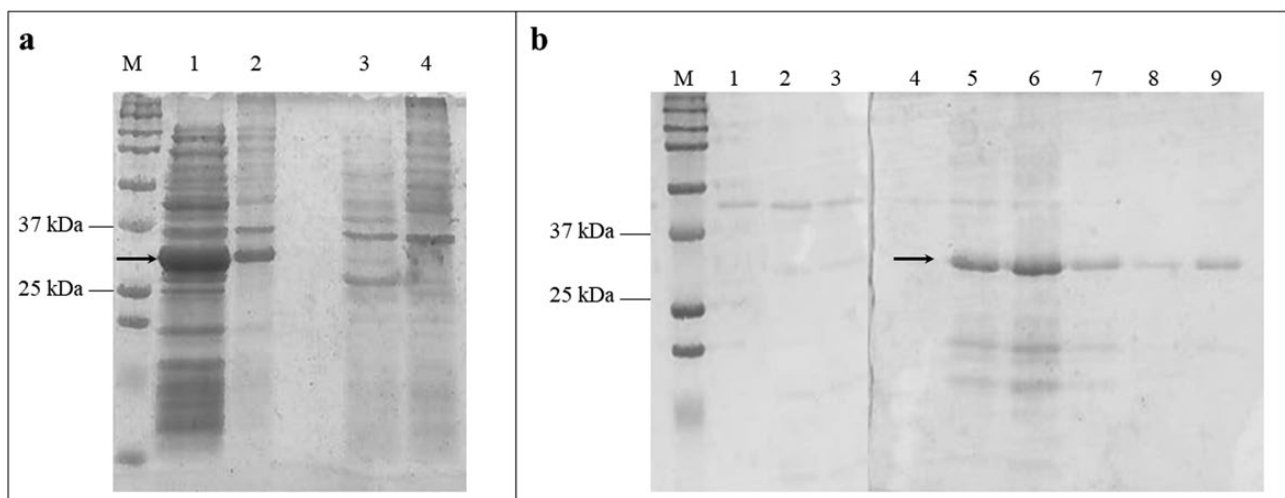
were performed on a single PG, in four technical replicates for each of the 10 biological replicates.

### Analysis of Protein Phosphorylation

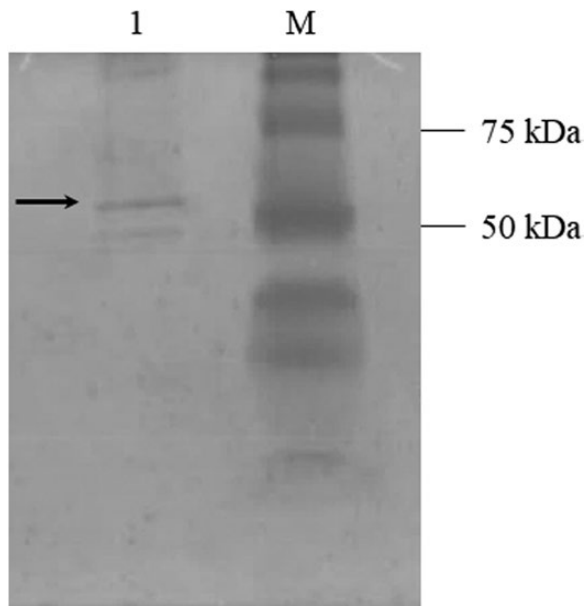
To verify the ability of the rPTTH to stimulate ecdysteroidogenesis through PI3K/Akt/TOR pathway, western blot analysis were performed on PGs previously incubated in Grace's insect medium (Sigma-Aldrich) and stimulated with 0.1 ng/ $\mu$ l rPTTH for 3 h at 25°C. A pool of 20 PGs for each condition (control and PGs stimulated by rPTTH) was incubated and lysed directly in Laemmli 2 $\times$  sample buffer (Laemmli 1970), allowing the inhibition of proteases and phosphatases. The extracted proteins were separated by a 10% polyacrylamide gel electrophoresis and transferred on a Whatman nitrocellulose membrane (Protran, Dassel, Germany). Specific antibodies were used to evaluate the phosphorylation of two TOR targets: anti-phospho-4E-BP (Cell Signaling, catalogue number 2855S, Danvers, MA) and anti-phospho-56K (Millipore, catalogue number 04-393, Temecula, CA).  $\alpha$ -Actin was used as an endogenous control using an anti- $\alpha$ -actin antibody (Abcam, catalogue number 75186, Cambridge, United Kingdom). All antibodies were diluted 1:1,000 in Tris-buffered saline added with 0.1% Tween-20 (TBS-T) with 5% bovine serum albumin, and the incubation was carried out for 16 h. Membranes were sequentially incubated with each of three antibodies. Goat anti-rabbit conjugated to horseradish peroxidase (Invitrogen), diluted 1:15,000 in TBS-T, was used as a secondary antibody. Enhanced chemiluminescence (ECL) (LiteAB Blot kit, Euroclone, Pavia, Italy) was used for the detection and signals were measured by Chemidoc<sup>TM</sup> MP System (Bio-Rad).

### Statistical Analysis

Enzyme immunoassay data were expressed as the mean  $\pm$  SEM of 10 independent biological replicates and evaluated by GraphPad Prism 6 software, version for Windows ([www.graphpad.com](http://www.graphpad.com)) (GraphPad Software, La Jolla, CA). For the analysis, a paired *t*-test was used to compare the mean amount of ecdysone produced, following the in vitro stimulation by recombinant hormone and brain extract. Differences compared with a control group and differences among



**Fig. 1.** Expression and purification of rPTTH. (a) Lane M: Bio-Rad Standard Kaleidoscope Protein Molecular Weight Marker; lane 1: supernatant from *E. coli* BL21 (DE3) cells, induced with 0.5-mM IPTG for 4 h at 37°C and lysed under denaturing conditions; lane 2: pellet from *E. coli* BL21 (DE3) cells, induced with 0.5-mM IPTG for 4 h at 37°C and lysed under denaturing conditions; lane 3: supernatant from not induced and lysed *E. coli* BL21 (DE3) cells; and lane 4: pellet from not induced and lysed *E. coli* BL21 (DE3) cells. (b) Lane M: Bio-Rad Standard Kaleidoscope Protein Molecular Weight Marker (Bio-Rad); Lanes 1–4: eluate with Elution buffer at pH 5.9; lanes 5–9: eluate with Elution buffer at pH 4.5.



**Fig. 2.** Native PAGE of rPTTH. Native PAGE showed the correct folding of recombinant PTTH. Lane M: Bio-Rad Standard Kaleidoscope Protein Molecular Weight Marker (Bio-Rad); lane 1: purified rPTTH (60 kDa).

increasing concentration of rPTTH were analyzed by analysis of variance, followed by Tukey's posthoc test.

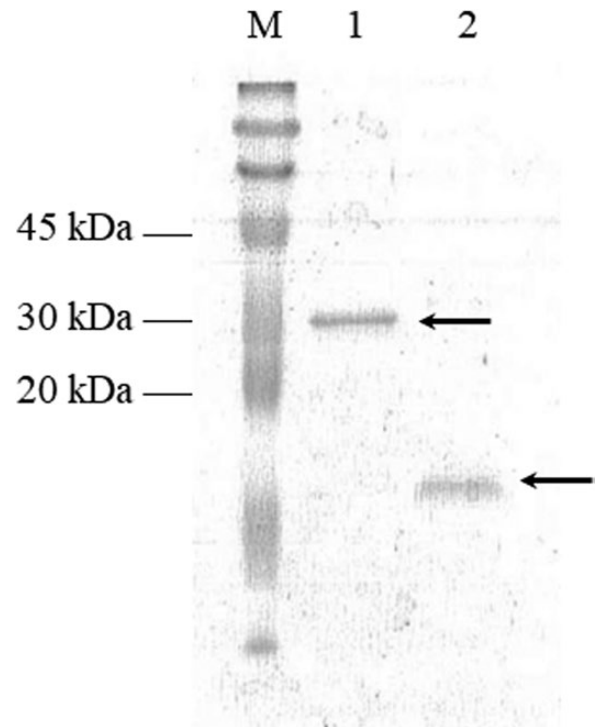
## Results

### Cloning, Expression, and Purification of rPTTH

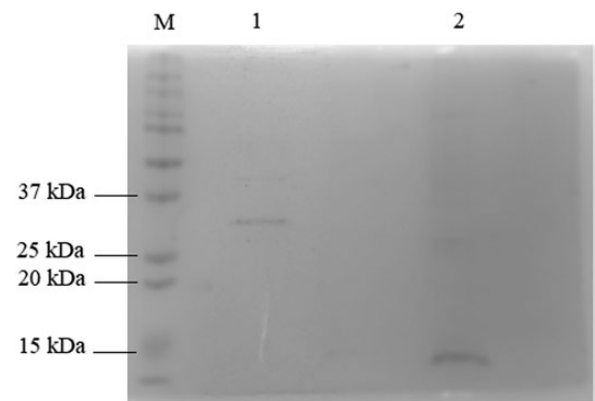
As a result of PCR amplification, a 368-bp fragment, coding for the active form of *H. virescens* PTTH, was purified and ligated into pET-32 Ek/LIC vector. The sequencing analysis confirmed that the amplicon was in frame under T7 promoter. rPTTH, with the expected molecular weight of 30 kDa, was detected both in supernatant and pellet, after *E. coli* BL21 (DE3) cells induction by IPTG (Fig. 1a, lanes 1 and 2). Although part of rPTTH was localized also in the inclusion bodies, it was mostly found in the soluble fraction, confirming its hydrophilic feature. The recombinant protein was purified using affinity chromatography on nickel resin Qiagen Ni peptide under pH gradient. rPTTH with the expected molecular weight of 30 kDa was present only in the elution fractions with Elution buffer at pH 4.5 (Fig. 1b, lanes 5–9) and not in elution fractions with Elution buffer at pH 5.9 (Fig. 1b, lanes 1–4). The correct refolding of rPTTH into the biologically active homodimeric form was analyzed by native PAGE (12.5%) that showed rPTTH with the expected molecular weight of 60 kDa (Fig. 2). Purified rPTTH was successfully digested by 0.01 units of thrombin/ $\mu$ g to remove the 14-kDa Trx and His tags and generate a 16-kDa peptide, corresponding to the mature rPTTH (13 kDa) with S tag (3k Da). The analysis of the sample by SDS-PAGE (15%) showed a single band corresponding to the protein of interest (Fig. 3). The correct refolding of the thrombin digested rPTTH into the biologically active homodimeric form and integrity were analyzed by SDS-PAGE (15%) in the presence and in the absence of  $\beta$ -mercaptoethanol (Fig. 4).

### Structural Characterization of rPTTH

The primary structure of purified intact rPTTH was verified by mass spectrometric procedures following essentially the mass mapping strategy. Reduced and carboxyamidomethylated rPTTH was



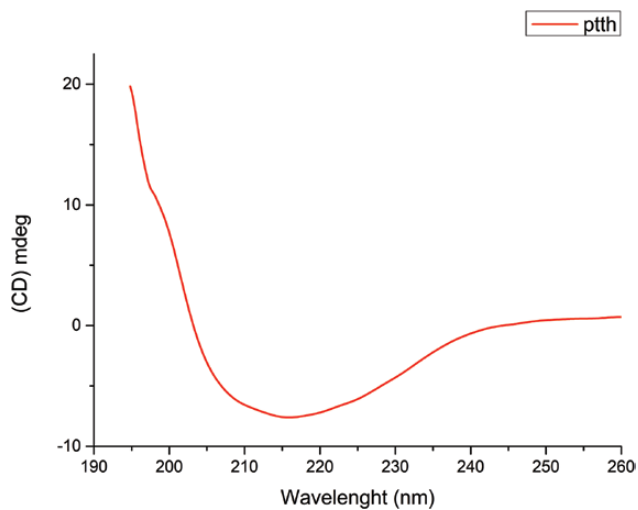
**Fig. 3.** SDS-PAGE of rPTTH digested with thrombin. rPTTH was digested by using 0.01 units of thrombin/ $\mu$ g of purified protein. The cleavage generated a 16-kDa peptide corresponding to the mature PTTH (13 kDa) and the S tag (3 kDa). Lane M: ColorBurst Electrophoresis Marker (Sigma-Aldrich); lane 1: rPTTH before thrombin digestion; lane 2: rPTTH after thrombin digestion.



**Fig. 4.** SDS-PAGE of the thrombin-digested rPTTH in the presence and in the absence of  $\beta$ -mercaptoethanol. SDS-PAGE showed the integrity of the thrombin digested recombinant PTTH. Lane M: Bio-Rad Precision Plus Protein All Blue Standards (Bio-Rad); lane 1: unreduced, refolded rPTTH (32 kDa); lane 2: rPTTH reduced by  $\beta$ -mercaptoethanol (16 kDa).

digested with trypsin and the resulting peptide mixture directly analyzed by both MALDI MS/MS and LC-MS/MS. The mass signals recorded in the spectra were mapped onto the anticipated sequence of rPTTH leading to a 78% coverage of the protein primary structure.

The secondary structure of rPTTH was evaluated by Circular Dichroism analysis. Protein (5  $\mu$ M) was dissolved in 10 mM sodium phosphate buffer, pH 7.4. The CD spectrum was recorded at 25°C and displayed a minimum at 216 nm typical of the beta-sheet structure as shown in Fig. 5. Deconvolution of the CD spectrum showed



**Fig. 5.** Analysis of rPTTH secondary structure. Circular dichroism spectrum of the PTTH sample in phosphate buffer pH 7.4 showing the minimum of absorbance at 216 nm.

that rPTTH has 36% beta-sheet structure, whereas 14% is associated with beta-turn structures.

#### Evaluation of BE and rPTTH In Vitro Activity

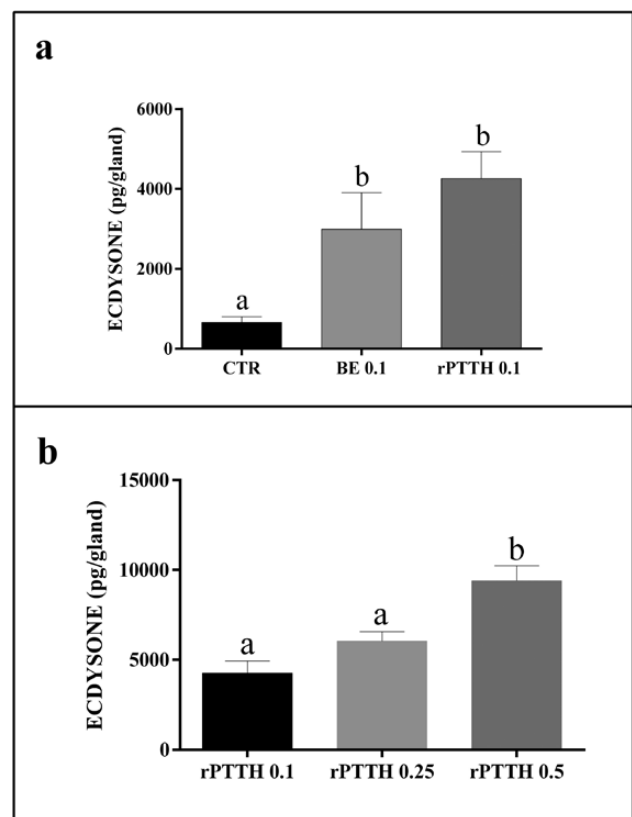
The evaluation of ecdysone titer produced in vitro and released by *H. virescens* PGs in different conditions was performed by EIA. Ecdysone in vitro secretion was enhanced by BE (0.1 brain equivalent/ $\mu$ l) ( $2,991.12 \pm 945.88$  pg/gland) and rPTTH (0.1 ng/ $\mu$ l) stimulation ( $4,263.96 \pm 1,348.38$  pg/gland), both statistically different from the control ( $660.84 \pm 208.98$  pg/gland) ( $F = 7.63$ ;  $P < 0.05$ ; Fig. 6a). In order to evaluate the biological activity of the recombinant hormone, PGs were also stimulated with different concentration of rPTTH (0.25 and 0.5 ng/ $\mu$ l). No statistically significant difference was measured between rPTTH 0.1 and 0.25 ng/ $\mu$ l stimulation ( $6,044.44 \pm 1911.42$  pg/gland); significant difference was observed between rPTTH 0.25 and 0.5 ng/ $\mu$ l stimulation ( $9,408.78 \pm 2,975.32$  pg/gland) ( $F = 14.54$ ;  $P < 0.0001$ ; Fig. 6b).

#### Phosphorylation of 4E-BP and S6K Proteins in PGs

To verify the influence of rPTTH on PI3K/Akt/TOR pathway in *H. virescens* ecdysteroidogenesis, western blot analysis was performed on PGs. The phosphorylation of the main targets of TOR kinase was detected using antibodies against phospho-4E-BP and phospho-S6K. The in vitro exposure of PGs explanted from 3 d (V3) old last (fifth) instar larvae to rPTTH enhanced the phosphorylation level of both 4E-BP and S6K proteins. No phosphorylation signal was detected in unstimulated PGs, incubated in Grace's insect medium (Fig. 7).

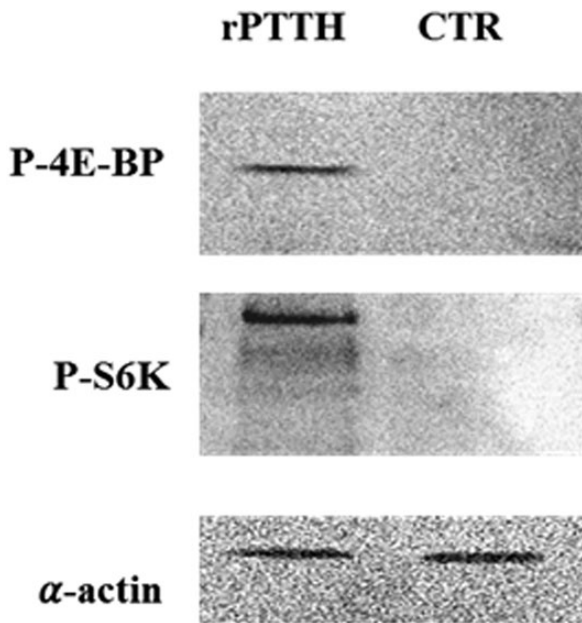
#### Discussion

The natural prothoracicotropic hormone (nPTTH) is a neuropeptide representing the main stimulatory factor on the PGs, capable of triggering the entire process of ecdysteroidogenesis (Huang et al. 2008). nPTTH is a brain extract component, along with other factors, such as insulin and prothoracicostatic peptide (PTSP), the natural antagonist of nPTTH (Gilbert 2012). The use of the brain extract that includes nPTTH could be deviating, as the different components effects are not easily evaluable; at certain times of the larval stage, the production of ecdysone could be also dependent on an



**Fig. 6.** In vitro effects of BE and rPTTH on ecdysone synthesis by PGs. PGs explanted from 3-d (V3) last (fifth) instar larvae were stimulated with BE (0.1 brain equivalent/ $\mu$ l) or rPTTH (0.1 ng/ $\mu$ l). Control glands (CTR) were incubated in Grace's insect medium (a). To evaluate the biological activity of rPTTH, PGs were stimulated with increasing concentration of the recombinant hormone (0.1, 0.25 and 0.5 ng/ $\mu$ l). (b) The ecdysone was determined by EIA after 3 h of incubation. Data are expressed as the mean concentrations of ecdysone (pg/gland)  $\pm$  SEM of  $n = 10$  experiments. Statistical analysis was performed by one-way analysis of variance followed by Tukey's posthoc test. Different letters indicate significant differences ( $P < 0.05$ ).

insulin-like factor. At increasing dose of nPTTH, the effect of PTSP could be prevalent, preventing the ecdysone production. Indeed, it is known that PTSP inhibits basal (Hua et al. 1999, Dedos et al. 2001) and nPTTH-stimulated (Hua et al. 1999) ecdysteroidogenesis in vitro in a dose-dependent manner. PTSP negatively regulates the effect of the neuropeptide on target organs, in order to modulate the physiological hormone response over the time (Gilbert et al. 2000). Moreover, although the active portion of nPTTH shows a high similarity among lepidopterans, the effect of the recombinant and natural hormone could be different: in *M. sexta* rPTTH and nPTTH show identical effects in terms of ecdysone synthesis (in a dose-, time-, and instar-dependent manner), cAMP levels and protein phosphorylation pattern (Gilbert et al. 2000); differently, in *Bombyx mori*, nPTTH, and rPTTH show different ability to stimulate ecdysteroidogenesis: the ecdysone titer measured after rPTTH stimulation is significantly lower than that obtained by nPTTH stimulation and does not consistently stimulate cAMP level in last (fifth) instar larvae and in the first day of pupae (Dedos et al. 1999). In previous studies (Scieuzo et al. 2018), we observed a consistent ability of crude brain extracts to stimulate ecdysteroidogenesis in *H. virescens* PGs. In this work, a rPTTH from *H. virescens* was expressed, purified and its primary and secondary structures were characterized to obtain a hormone useful to perform in vitro tests, in a standard and repeatable manner. Our results derived from the SDS-PAGE analysis of purified rPTTH



**Fig. 7.** Phosphorylation of TOR target proteins in PGs. PGs from 3-d old last (fifth) instar larvae were stimulated *in vitro*, in a Grace's insect medium, added with 0.1 ng/ $\mu$ l of rPTTH. Unstimulated PGs were incubated in Grace's insect medium. Incubation was maintained for 3 h at 25°C in each condition. Glands lysed in Laemmli 2x sample buffer were analyzed by western blot using antibodies against phospho-4E-BP (20 kDa) and phospho-S6K (70 kDa). Each lane represents the equivalent of 20 PGs.  $\alpha$ -Actin (42 kDa) was used as endogenous control.

from *H. virescens*, in the presence and in the absence of the reducing agent  $\beta$ -mercaptoethanol, confirm literature data concerning the homodimeric nature of the biologically active insect PTTH (Kataoka et al. 1991, Ishibashi et al. 1994, Shionoya et al. 2003). rPTTH from *H. virescens* has a secondary structure composed of 36% beta-sheet and 14% of beta turn structures. This agrees with the rPTTH secondary structure observed in other insect species (Noguti et al. 1995, Zhang and Denlinger 2011). rPTTH activity on PGs was evaluated by *in vitro* tests in comparison to the brain extract (BE) mixture, demonstrating that rPTTH from *H. virescens* and nPTTH found in the BE have comparable effects. Data from experiments performed with BE stimulation at the concentration of 0.1 brain equivalent/ $\mu$ L (Pennacchio et al. 1997, 1998; Scieuzo et al. 2018) revealed that rPTTH has equivalent effects on *in vitro* PG stimulation and on the phosphorylation of the two TOR pathway targets: 4E-BP and S6K. Our results confirm literature data, indicating that nPTTH, as a component of the brain extract mixture, increases the *in vitro* production and secretion of ecdysone in *H. virescens* PGs (Pennacchio et al. 1997). Our experiments highlight that *H. virescens* rPTTH has a similar effect in comparison to the brain extract in the stimulation of PGs biosynthetic activity. No statistically significant differences among samples differently stimulated (nPTTH or rPTTH) were observed. Furthermore, to investigate the rPTTH quantitative biological activity, the ecdysone produced *in vitro* and released by *H. virescens* PGs was measured, using increasing concentration of the purified recombinant hormone. Overall, the *in vitro* activity of increasing concentration of rPTTH displays the ability to stimulate PGs to produce and secrete increasing amount of ecdysone, overcoming the potential inhibitory effect observed following the natural hormone stimulation. This allows the standardization and reproducibility of experiments aimed to study ecdysteroidogenesis. Our results

show that, similarly to the natural hormone, rPTTH acts through a transduction cascade that includes multiple phosphorylations of the two main targets of TOR pathway, 4E-BP and S6K. The possibility to use the recombinant PTTH for further experiments aimed to investigate the mechanisms of ecdysteroidogenesis is of considerable importance. Comparability in the use of *H. virescens* rPTTH and crude brain extract strengthen data deriving from previous research, suggesting that the effect is due to a greater extent to natural PTTH, despite the presence of other stimulatory or inhibitory factors.

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