Characterization of a cell death-inducing endonuclease-like venom protein from
the parasitoid wasp Pteromalus puparum (Hymenoptera: Pteromalidae)

RUNNING TITLE: An endonuclease-like venom protein induces cell death

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ABSTRACT

BACKGROUND: Parasitoid wasps are valuable natural enemies for controlling pests. To ensure successful parasitism, these wasps inject venoms along with their eggs that are deposited either into or on their hosts. Parasitoid venoms regulate host behaviors, development, metabolism, and immune responses. *Pteromalus puparum* is a pupal endoparasitoid that parasitizes a number of butterflies, including the worldwide pest cabbage butterfly, *Pieris rapae*. Venom from *P. puparum* has a variety of effects on host hemocytes, including alteration of absolute and relative hemocyte counts, and inhibition of hemocyte spreading and encapsulation. In particular, *P. puparum* venom causes hemocyte cell death *in vivo* and *in vitro*.

RESULTS: Using assay-guided chromatography, a cell death-inducing venom fraction was identified and defined as *P. puparum* endonuclease-like venom protein (*PpENVP*). It belongs to the DNA/RNA non-specific endonuclease family, which contains two conserved endonuclease activation sites. We analyzed its expression profiles and demonstrated that *PpENVP* inhibits gene expression in transfected cells relying on two activation sites. However, RNA interference of *PpENVP* did not significantly reduce *P. puparum* venom cytotoxicity, suggesting that *PpENVP* may not be the sole cytotoxic factor present.

CONCLUSION: Our results provide novel insight into the function of the *P. puparum* venom cocktail and identify a promising insecticide candidate endonuclease that targets insect hemocytes.

KEYWORDS: *Pteromalus puparum*, Endonuclease, Venom, Hemocyte, Encapsulation, Cell death, Cytotoxicity, *PpENVP*
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INTRODUCTION

Parasitoid wasps make up a large portion of the Hymenoptera, as more than 100,000 species have been described and more than 600,000 species are estimated. They usually lay eggs in or on the body of various invertebrate hosts, and after these eggs hatch the wasp larvae consume host tissues throughout development causing host death. Because of their unique life cycle, parasitoid wasps can effectively suppress invertebrate pest populations. Numerous species have been used as biocontrol agents to control agricultural and sanitary pests in the fields.

During oviposition, parasitoid wasps inject virulence factors into their hosts to enhance the success of parasitism. These virulence factors include but are not limited to virulence proteins, polydnaviruses (PDV), teratocytes, virus-like particles/filaments (VLP/VLF), and ovarian proteins. Since venom exists in all reported parasitic wasp species, it is thought to be essential to the parasitoid lifestyle. Because of the rapid development of transcriptomics and proteomics in recent years, venom proteins from numerous parasitoids have been identified, and it is clear that their numbers and functions vary dramatically across species. Venom proteins have been shown to control host behaviors, host development, metabolism, and immune responses. However, the functions of most identified venom proteins have not been experimentally investigated and very few have been shown to have cytotoxic activity.

Many toxic proteins, including those from parasitoid venoms, have been successfully developed for application in the field as pesticides. For example, expression of Egf 1.0 from the PDV of the parasitoid wasp Microplitis demolitor in the Semliki Forest virus successfully enhanced its lethal effect on the mosquito Aedes aegypti by inhibiting phenoloxidase activity. Expression of the secretory protein TSP14 from the teratocytes of the wasp M. croceipes Cresson in transgenic tobacco plants increased its resistance against the tobacco budworm Heliothis virescens (Fabricius) and tobacco hornworm Manduca sexta (Linnaeus). Expression of TnBVANK1 from the PDV of Toxoneuron nigriceps in transgenic tobacco plants increased its resistance against the cotton leafworm Spodoptera littoralis. Thus, there is a general understanding that venom proteins with cytotoxic activity are promising insecticidal
candidates that might be transferred into crops or insect pathogens to enhance agricultural production\textsuperscript{20, 21}.

The pupal endoparasitoid \textit{Pteromalus puparum} (Hymenoptera: Pteromalidae) is common throughout the world\textsuperscript{22}. It is used as a biocontrol agent against the cabbage butterfly, \textit{Pieris rapae} (Lepidoptera: Pieridae), a worldwide pest of cruciferous crops\textsuperscript{23}. During oviposition, \textit{P. puparum} injects venom into its host pupae as the key virulence factor, that inhibits host hemocyte spreading and encapsulation\textsuperscript{24-27}. Intriguingly, \textit{P. puparum} venom is capable of inducing hemocyte death\textsuperscript{24} and its adverse effects on hemocytes were not only found in its natural hosts but also in some non-target insects and cultured insect cells\textsuperscript{25}. Combining \textit{P. puparum} transcriptomics datasets with proteomic analyses of its venom, we previously identified 70 venom proteins/peptides in \textit{P. puparum}\textsuperscript{28, 29}. However, the particular venom protein(s) responsible for host hemocyte cell death remains unknown.

Here, we identify an endonuclease-like venom protein (ENVP) as a cell death-inducing protein in \textit{P. puparum} venom, and describe the protein’s activation sites and assay its cytotoxicity in a variety of experiments. We find that \textit{PpENVP} is a promising insecticidal candidate gene and suggest it be further developed for pest biocontrol systems in the future. Use of such natural toxins can enhance the quantity and quality of agricultural products while reducing the negative environmental impacts of traditional insect control practices.

\section*{2 MATERIALS AND METHODS}

\subsection*{2.1 Insect rearing}

\textit{Pieris rapae} larvae were collected from the experimental cabbage fields at Zhejiang University, Hangzhou, China, and subsequently fed on fresh cabbage leaves and maintained in laboratory incubators at 25 ± 1°C with a light/dark cycle of 10/14 hrs until pupated. The laboratory \textit{P. peparum} colony was raised under the same conditions and fed on 20\% (v/v) honey/water solution to extend their life spans. For parasitism, each \textit{P. rapae} host was exposed to one mated female wasp \textit{P. puparum} in a glass vial (18 × 82 mm). The parasitized hosts were kept at the same conditions mentioned above until parasitoid wasps emerged.
2.2 Venom protein extraction

Venom from *P. puparum* was prepared by the methods described by Wu et al. 27. ~1,000 collected venom glands and reservoirs were transferred to cold sterilized 1.5 ml Eppendorf tubes with 10 mM phosphate-buffered saline (PBS) and then spun at 16,000 g for 20 min at 4°C. The supernatant was collected and diluted using PBS to a final concentration of 1 venom reservoir equivalent (VRE)/µl. Since some protease inhibitors have been reported to inhibit the cytotoxicity of *Nasonia* venom 16, no protease inhibitor was used in our experiments. To extract proteins from wasp individuals or different tissues (head, thorax, carcass, ovary, venom gland of 2-day old *P. puparum* females), samples were collected into cold centrifuge tubes containing PBS, homogenized using a grinding rod, and spun at 16,000 g for 20 min at 4°C) to remove the cuticle fragments. Extracted proteins were used immediately or stored at -80°C.

2.3 Detection of cell death using the CellTox Green Cytotoxicity Assay

Hi-5 or Sf-9 cell lines at 60% confluence were aliquoted in 15 µl volumes into 384-well plates. Venom from the wild-type adults or those with *PpENVP/eGFP* knockdown treatment was incubated with cells overnight respectively. Following the protocol of the CellTox™ Green Cytotoxicity Assay Kit, CellTox Green Dye and Assay Buffer were mixed to make 2 × reagent. After adding an appropriate volume of reagent, we incubated cells in darkness at 25°C for different time durations. Cell spreading was observed using a Nikon ECLIPSE TS100 microscope, and fluorescence was measured using Thermo Scientific™ Varioskan™ Flash at 490 nm excitation and 525 nm emission. Three replicates were performed for each treatment.

2.4 Fractionation of venom proteins

Venom components were separated using anion-exchange chromatography followed by size exclusion chromatography. For anion-exchange chromatography, a similar process was applied as described previously 15. Briefly, venom proteins from ~1000 venom reservoirs were loaded on Bio SAX column (5 µm, 4.6 × 50 mm, Agilent, USA) using a Biologic Duo-Flow high-performance chromatography system (Bio-rad, Germany). Proteins were eluted at a flow rate of 0.5 ml/min with a gradient of
buffer B (25 mM Tris-HCl, 1 M NaCl, pH = 7.5) against buffer A (25 mM Tris-HCl, pH = 7.5). Protein level was monitored by absorbance at 280 nm. Fractions of 200 µl were collected in a deep well plate (Eppendorf, Germany) using the BioLogic BioFrac fraction collector (Bio-rad, Germany), then desalted using Zeba spin desalting plates (Thermo Scientific, USA). Fractions 56 and 57, which showed the highest cytotoxicity on Hi-5 cells, were pooled for further size exclusion chromatography using Agilent Bio SEC columns (3 µm, 300 A, 4.6 × 300 mm) at a flow rate of 0.2 ml/min using PBS as the running buffer. Protein level was monitored by absorbance at 214 nm. The fraction with cytotoxicity was pooled and subjected to SDS-PAGE.

2.5 Identification of candidate cytotoxic venom proteins

A single fraction was run through an Easy-nLC HPLC system (Thermo Scientific, USA) followed by a Q-Exactive mass spectrometer (Thermo Finnigan, USA) as previously described. Briefly, the isolated protein from the cell death-inducing fraction was digested by trypsin using the FASP (Filter-aided sample preparation) method, then loaded on Thermo scientific EASY columns (5 µm, 2 cm × 100 µm, C18) and separated with a flow rate of 250 nl/min on another Thermo scientific EASY column (3 µm, 75 µm × 100 mm, C18). Buffer A was water with 0.1% formic acid, buffer B was 84% acetonitrile with 0.1% formic acid, and the gradient went from 0% to 50% buffer B in 50 min, then from 50% to 100% buffer B in 4 min. The charge-to-mass ratios of peptides were collected 10 times after every full scan. Raw data files were generated by the Xcalibur software (Thermo Scientific, USA) and processed using Proteome Discoverer v 1.4.1.14 (Thermo Scientific, USA). The resulting MS/MS spectra were searched against the translated *P. puparum* transcriptomic database (GenBank accession ID: GRECT01000000) using Mascot software version 2.2. The search criteria was as follow: Fragment mass tolerance= 0.1 Da; Peptide mass tolerance= 20 ppm; Max missed cleavages= 1. Cysteine carbamidomethylation and methionine oxidation were set as fixed and variable modifications, respectively. The search result filter was selected as score ≥ 20. MS data of the protein identification was shown in the supplementary file 1. This part of the work was done by the Shanghai Applied Protein Technology Co., Ltd (Shanghai, China).
2.6 RNA extraction, cDNA synthesis, and RT-qPCR

Tissues from 2-day old *P. puparum* female wasps (head, thorax, carcass, ovary, venom gland) were collected, washed, and pooled into separate 1.5 ml Eppendorf tubes. TRIzol reagent (Invitrogen, USA) was used to extract total RNA from each tissue sample. 1 μg of total RNA was used for each sample in the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China) to generate purified cDNA. RT-qPCR was performed using the Bio-Rad CFX 96 Real-Time Detection System (Bio-Rad, Hercules, CA, USA) with SYBR Green Supermix Kits (Takara, Japan). Thermal cycling conditions were: 94°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 30 s. The 2^{\Delta\Delta CT} method was used for quantification after normalization using the 18s gene as an internal control. Primers used for RT-PCR (*PpENVP*-qPCR-sp and *PpENVP*-qPCR-ap) were designed using the website Primer 3 (Table S1). Each treatment group was replicated at least three times.

2.7 Gene cloning

Primers (*PpENVP*-sp and *PpENVP*-ap) were designed covering full-length *PpENVP* cDNA using Primer 5 software (Version 5.0). LA Taq DNA polymerase (Takara Biomedical Technology Co., Ltd., Beijing, China) was used to perform PCR using the synthesized cDNA as a template. The reaction conditions were set up according to the user manual. PCR products were purified and cloned into the pGEM® T-easy vector (Promega, Beijing, China). Sanger sequencing for construct sequence confirmation was performed by Biosuno Company (Shanghai, China). Open reading frames (ORFs), molecular weights, and amino acid sequences of *PpENVP* were estimated using the DNA Star software package (Version 5.02). In further experiments, the *PpENVP* gene was sub-cloned into different vectors, namely pET-28a, pGEX-4T-2, pFastBac-HTB, pIZT/V5-his and pIEX-1. The recombinant plasmids were generated using the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China). All primers used for gene amplification are listed in Table S1.

2.8 Bacterial expression system and production of an antibody against *PpENVP*

Linear pET-28a and pGEX-4T-2 vectors were generated by BamHI and XhoI (TaKaRa, Dalian, China) restrictions, and used to construct recombinant *PpENVP*. 
The combination of *Escherichia coli* strain BL21(DE3) and pET-28a vector allowed recombinant mature *PpENVP* to be expressed in inclusion bodies in the autoinduction medium \(^{32}\) at 30 °C. These inclusion bodies were washed using Inclusion Body Purgation Buffer (Sangon Biotech, China), and rabbit polyclonal antibodies were produced against the inclusion bodies and then purified using the Montage Antibody Purification kit (Millipore, Billerica, MA). *PpENVP* antibody production and purification were conducted by HuaBio Co., Ltd (Hangzhou, China).

### 2.9 SDS-PAGE and Western blot

Protein concentration was determined by the Bradford method \(^{33}\). Each protein sample (~20 µg) was run on a 12% SDS-polyacrylamide gel (SDS-PAGE) with Coomassie blue staining. Immunoblotting analysis (Western blot) was performed using the rabbit polyclonal antibody against *PpENVP*, the rabbit monoclonal antibody against His-tag, and the rabbit monoclonal antibody against Actin (GeneScript, Inc) as primary antibodies (diluted 1 : 2500), and the goat anti-rabbit IgG-horseradish peroxidase conjugate (TransGen Biotech, China; diluted 1 : 5000) as the secondary antibody.

### 2.10 Generating *PpENVP* mutant constructs

Mutants for the *PpENVP* gene were constructed as follows. The first PCR step was achieved for each mutant using two sets of primers: (i) the common *PpENVP* forward primer (*PpENVP*-pIEX-sp) and a specific reverse primer for each mutant: *PpENVP*-M1-ap for the water activation site mutant, and *PpENVP*-M2-ap for the ion binding activation site mutant; (ii) the common *PpENVP* reverse primer (*PpENVP*-pIEX-ap) and a specific forward primer for each mutant: *PpENVP*-M1-sp for the water activation site mutant, and *PpENVP*-M2-sp for the ion binding activation site mutant. In the second step, two PCR-amplified fragments for each mutant were processed for homologous recombination using the ClonExpress MultiS One Step Cloning Kit (Vazyme biotech co., Ltd, Nanjing, China). For the double activation site mutant (M12), a second round of PCR was run using the DNA fragment corresponding to M1 or M2 mutant as a template. Finally, the three *PpENVP* mutant constructs (as well as eGFP and CAT genes) were sub-cloned into the pIEX-1 vector. All construct sequences were confirmed by Sanger sequencing. All primers used for generating mutant constructs were designed using Primer 5.0 software and are listed in Table S1.
2.11 Insect expression system and co-expression with eGFP

For the eGFP co-expression experiment, 300ng of pIEX-eGFP construct was first mixed with Grace Insect Medium (Thermo Scientific, USA) up to 40µl and added to single wells of a 96-well plate. 100 ng of pIEX-\(Pp\)ENVP wild type or mutant constructs (as well as the control pIEX-CAT) were then applied individually to each eGFP well depending on the treatment. The mixed constructs were co-transfected into Hi-5 cells following the FuGENE® HD Transfection Reagent (Promega Co., USA) protocol, where 2 µl reagent was used for each mixed sample. After gently mixing, we incubated the treated cells at 25°C for 48 hrs, and then assayed eGFP expression by measuring GFP fluorescent intensity (488 nm excitation and 510 nm emission).

For Western blot analysis, cells were washed with PBS 3 times and lysed with I-PERT™ Insect Cell Protein Extraction Reagent (Thermo Scientific, USA). Extracted proteins were collected and stored at -80°C.

2.12 Drosophila embryo microinjection

\(Pp\)ENVP and eGFP genes were each sub-cloned into the pUAST-attB (digested by EcoRI and Kpnl) and pBID-attB (digested by EcoRI and Xhol) vectors using two pairs of primers: \(Pp\)ENVP-pUAST-sp and \(Pp\)ENVP-pUAST-ap, and \(Pp\)ENVP-pBID-sp and \(Pp\)ENVP-pBID-ap. Transgenic lines were expected to be generated by injecting Qiagen-purified plasmid DNA into embryos of \(D.\) melanogaster strain \(ywR13s\) (\(yw;\) sp/Cyo; MKRS/TM2). Purification of plasmids, injection of \(Drosophila\) embryos, and the counting of the hatched embryos were performed by the Shanghai Institute of Biochemistry and Cell Biology, CAS (Shanghai, China).

2.13 RNA interference

For synthesis of dsRNA, \(Pp\)ENVP and eGFP plasmids were used as template DNA to perform PCR. These sequence-confirmed PCR products were purified and used as templates for \textit{in vitro} double-strand RNA (dsRNA) synthesis using the MEGAscript T7 Transcription Kit (Ambion, Austin, TX). All primers used were designed with an added T7 promoter adaptor (\(Pp\)ENVP-T7-sp and \(Pp\)ENVP-T7-ap, Table S1). Following the kit instructions, dsRNA was synthesized and then purified by phenol/chloroform extraction and isopropanol precipitation, and then dissolved in
RNase-free ddH₂O and quantified using a NanoDrop 2000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). We sorted yellow-stage female wasp pupae collected from one butterfly pupa into two groups: one for injection of PpENVP dsRNA, the other for injection of eGFP dsRNA. 50 nl of dsRNA (~3000 ng/nl) was injected into each wasp pupa. Venom from newly emerged female wasps was collected and used for RNAi efficiency validation in SDS-PAGE and Western blot experiments. The cytotoxic activity of PpENVP knockdown venom was assayed using the CellTox™ Green Cytotoxicity Assay Kit.

2.14 Statistical analyses
The fluorescence intensity data were analyzed using one-way ANOVA and Tukey’s or LSD’s multiple comparison test. These statistical calculations were run on Data Processing System software (version 14.50). Chi-Square tests (2 × 2) were run on IBM® SPSS software (version 22).

3 RESULTS
3.1 Venom fractionation and identification of the venom protein PpENVP
Venom from ~1,000 P. puparum venom reservoirs was fractionated using anion-exchange chromatography (Fig. 1A). Each fraction was incubated with Hi-5 cells (derived from the cabbage looper, Trichoplusia ni), and a CellTox™ Green Cytotoxicity Assay Kit was used to detect cell death. This kit uses an asymmetric cyanine dye to bind DNA in compromised cells so that dead cells are easily identified with fluorescence. Two contiguous fractions # 56 and # 57 were found with the highest cytotoxicity among all 95 fractions from the anion-exchange chromatography (Fig. 1A red arrow). To reduce interference from other fractions, only these two contiguous fractions were mixed and subjected to size exclusion chromatography. A single fraction derived from the size exclusion trial significantly induced cell death (Fig. 1B) and was called cell death-inducing fraction (CDIF). After the incubation with CDIF, Hi-5 cells significantly took on a rounded configuration and were detected as dead cells using the cytotoxicity assay Kit (Fig. 1D). Furthermore, this CDIF presented as a single band with a molecular weight of ~65 kDa in the SDS-PAGE gel (Fig. 1C).
To identify the corresponding cytotoxic venom protein, the isolated cytotoxic fraction was subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS). After searching against the translated *P. puparum* transcriptomic database, a predicted venom gene comp42418_c0 was identified with 14 matched peptides (coverage 30.38%, Fig. 2A). This gene contains an open reading frame of 1,539 bp (coding for 513 amino acids). Using the BLASTP algorithm from the National Center for Biotechnology Information (NCBI) website, the best hit found was an endonuclease-like venom protein precursor from *Nasonia vitripennis* (ref [NP_001155087.1]), which preferentially matched with total score = 613, query cover = 92%, E-value = 0.0 and identity = 62%. With a recognized Endonuclease_NS domain (pfam01223), this gene was identified as belonging to the DNA/RNA non-specific endonuclease family, and was thus named *P. puparum* endonuclease-like venom protein (*Pp*ENVP). Additionally, a spectrum of the representative peptide sequence identified from *Pp*ENVP is shown in Fig. 2B.

*Pp*ENVP has a predicted signal peptide 23 amino acids in length and an estimated molecular weight of 53.5 kDa. BLASTP results show that its Endonuclease_NS domain begins at the 187th residue and ends at the 415th residue. The typical structure of endonucleases has been analyzed in *Serratia marcescens* 34, *Caenorhabditis elegans* 35, *Drosophila melanogaster* 36 and *Bos taurus* 37. A well-characterized member of endonucleases, the human endonuclease G (EndoG), usually has a highly folded ββα-Me-finger motif and two conserved activation sites 36, including a histidine residue as a water activation site and an asparagine residue as Mg$^{2+}$ ion binding site 37. In *Pp*ENVP these two activation sites are located at the 291st residue and the 321st residue, respectively (Fig. 2A).

### 3.2 Expression profile of *Pp*ENVP

To verify whether *Pp*ENVP is a venom protein, we investigated its expression pattern using RT-qPCR and Western blot. RNA samples were collected from five different tissues in *P. puparum* adult females (head, thorax, carcass, ovary, and venom gland). The results showed that *Pp*ENVP is specifically expressed in *P. puparum* venom glands as its relative expression level in venom glands was significantly higher than in other tissues (p < 0.001, Fig. 2C). Similarly, the Western blot results showed that
ENVP protein is found mainly in *P. puparum* females rather than males, and specifically in their venom reservoirs (Fig. 2D). The detected molecular weight of *Pp*ENVP was ~65 kDa, which is larger than the estimated molecular weight (53.5 kDa). This is a common phenomenon for venom proteins and is probably due to post-translational modifications (PTMs), which modify the existing function group or introduce a new one to extend the chemical repertoire of standard amino acids and lead to the protein molecular weight change. In addition, a predicted N (aspartagine)-linked glycosylation site was located at the 31st residue using the PTM prediction tool named ELM prediction (http://elm.eu.org). Besides of this ~65 kDa band, two additional protein bands between 130 and 170 kDa were observed in our venom reservoir column. They may be caused by several reasons, including the non-specific binding of the rabbit polyclonal antibody against *Pp*ENVP, the multimers forming of target protein, or the multiple modified forms of *Pp*ENVP. Our results strongly indicated that *Pp*ENVP is a venom-specific protein in *P. puparum*.

### 3.3 Functional analysis of recombinant *Pp*ENVP

To express *Pp*ENVP *in vitro*, we used both bacterial and insect cell expression systems. The *Pp*ENVP gene was cloned into the bacterial plasmids pET-28a and pGEX-4T-2, and into the eukaryotic vectors pFastBac-HTB, pIIZT/V5-his, and pIEX-1. In general, no transfected cells producing *Pp*ENVP were found, with the exception that *Pp*ENVP was expressed in bacterial inclusion bodies when the pET28a vector was used. These data suggest that *Pp*ENVP protein has general cytotoxic activity against bacterial and insect cells. Hence, we modified two sites thought to be critical to endonuclease function, both separately and in combination. An Asparagine residue from the water activation site was replaced by Alanine, generating *Pp*ENVP mutant1 (M1); A Histidine residue from the ion-binding site was replaced by Alanine, generating *Pp*ENVP mutant2 (M2). For the mutant with both sites replaced, it was named *Pp*ENVP mutant12 (M12). After cloning those three *Pp*ENVP mutant genotypes into the vector pIEX-1, the constructed plasmids were transfected into Sf-9 cells (derived from the fall armyworm, *Spodoptera frugiperda*). However, no *Pp*ENVP protein was produced by the transfected cells, as determined by Western blot.
Although the difficulty in obtaining *PpENVP* protein hindered direct investigation of its function, we conducted further transfection experiments to assay its toxicity indirectly. GFP (eGFP), *PpENVP* wild-type (WT), *PpENVP* mutants (M1, M2, M12), and catalase (CAT) sequences were inserted into the vector pIEX-1. The eGFP plasmid was co-transfected with the other plasmid constructs into Hi-5 cells, and the GFP fluorescence intensity of each combination was assayed. Using one-way ANOVA and least significant difference (LSD) multiple comparison tests with a significance threshold of *p* < 0.05, we found that the wild-type *PpENVP* protein significantly reduced the expression of eGFP compared to the positive control CAT construct (Fig. 3A, B). These data imply that *PpENVP* is capable of inducing the death of transfected cells or inhibiting gene expression in cells. Intriguingly, while the two *PpENVP* mutants with only one activity site replaced (M1 and M2) both weakened eGFP expression to the same extent that wild type *PpENVP* did, M12 with two modified activity sites failed to reduce eGFP expression (Fig. 3A, B). These data indicate that the cytotoxic activity of *PpENVP* is dependent on both endonuclease activity sites. The eGFP fluorescence data were validated using Western blots targeting the eGFP His tag (Fig. 3C).

Additionally, we constructed *D. melanogaster* transgene insertion plasmids (pUAST-attB) containing *PpENVP* and eGFP constructs. The majority of embryos (96.0%) injected with pUAST-attB-*PpENVP* plasmid failed to hatch, and none of the hatched larvae successfully eclosed. However, 17.5% of the embryos injected with the pUAST-attB-eGFP control plasmid successfully hatched, and their eclosion rate was 28.57%. In theory, the UAS transgene constructs should only be expressed in the presence of the Gal4 transcription factor, but we assume that leaky transgene expression is responsible for the embryonic and larval lethality observed. For this reason, a second vector (pBID-attB) was also used but the results were much the same. *D. melanogaster* embryos injected with purified pBID-attB-*PpENVP* vector barely hatched, and none made it through eclosion. On the other hand, 40.0% of embryos injected with the pBID-attB-eGFP vector hatched and survived well. We performed a 2 × 2 Chi-Square test for each vector set and found that *PpENVP* constructs induced significantly higher mortality in *D. melanogaster* embryos than the control (eGFP) constructs (Table 1).
3.4 RNAi knockdown of the *P. puparum* PpENVP gene

We used RNA interference (RNAi) to down-regulate the expression of *PpENVP* in *P. puparum*, and then assayed the cytotoxic activity of whole venom. Double-stranded RNAs (dsRNAs) designed for *PpENVP* and eGFP were injected into *P. puparum* female yellow-stage pupae. Venom glands were removed from 2-day female adults for RT-qPCR quantification of venom gene expression, while venom proteins were extracted for use in SDS-PAGE and Western blot quantification experiments. The RT-qPCR results showed that RNAi significantly down-regulated *PpENVP* expression (*p* < 0.01, Fig. 4A). On the SDS-PAGE gel, the ~65kDa *PpENVP* band had a lighter color when knocked down compared to the control eGFP RNAi sample (Fig. 4B). Similarly, Western blots using an anti-*PpENVP* antibody showed a stronger ~65kDa band in the eGFP knockdown treatments than in the *PpENVP* knockdown treatments. Altogether, these data show that *PpENVP* can be successfully knocked down by RNAi.

Based on the successful RNAi knockdown, we extracted venoms from *PpENVP* RNAi and eGFP RNAi female wasps to incubate with Hi-5 cells, then measured cell mortality using the CellTox™ Green Cytotoxicity Assay. For Hi-5 cells incubated with 1/16, 1/8, 1/4/ and 1/2 venom reservoir equivalent (0.0625 VRE/μl, 0.125 VRE/μl, 0.25 VRE/μl, and 0.5 VRE/μl) for 8 hrs, fluorescent intensity (cell death) increased with increasing concentration but there was no significant difference between the *PpENVP* and eGFP knockdown samples (Fig. 4C) at each venom concentration level. Time-course quantifications of fluorescence intensity in Hi-5 cells treated with different venom concentrations were also displayed (Fig. S1). No difference between the *PpENVP* and eGFP knockdown samples was found once again. These data show that loss of *PpENVP* in whole venom does not mitigate cell toxicity, presumably because there are other redundant cytotoxic components in *P. puparum* venom.

4 DISCUSSION

In response to the venom of the pupal endoparasitoid *P. puparum*, host hemocytes increase in number but also lose the ability to spread over and encapsulate foreign
objects such as the wasp egg. Although no marked alteration of hemocyte cytoskeletons is observed in response to venom, hemocytes rapidly take on a rounded configuration after treatment, and eventually their membranes are ruptured and they die \cite{25,38}. To investigate the venom components causing cell death, we isolated a fraction from *P. puparum* venom that shows cytotoxicity *in vitro*. This cell death-inducing fraction contained only one common protein, an endonuclease-like venom protein that we named *Pp*ENVP. *Pp*ENVP belongs to the DNA/RNA non-specific endonuclease family. Previously, an increasing number of endonucleases have been identified in the venom of parasitic wasps \cite{39,40,41,42}, but few have been functionally characterized.

The DNA/RNA non-specific endonuclease family is found across prokaryotic and eukaryotic organisms and its members are capable of cleaving double-stranded and single-stranded nucleic acids non-specifically \cite{43}. For example, the endonuclease G (EndoG) was first described to cause apoptosis in human Hela cells \cite{46} and is highly conserved among eukaryotes \cite{36,37,45}. It is now known to cause both apoptotic and non-apoptotic programmed cell death across eukaryotic cells \cite{46}. When hemocytes were treated with *P. puparum* venom, no sign of apoptosis such as cell membrane blebbing was observed, and thus we assume that cell death caused by *P. puparum* venom is controlled by a non-apoptotic mechanism. Pursuing experiments to reveal the exact mechanism by which *Pp*ENVP induces cell death would be useful.

However, several of our attempts to express recombinant *Pp*ENVP protein using *in vitro* protein expression systems failed. We surmised that the transfected cells were being impaired or killed by the *Pp*ENVP protein, and to test this hypothesis we co-transfected *Pp*ENVP and eGFP plasmids into Hi-5 cells. While eGFP constructs were expressed at appreciable levels, co-transfection with *Pp*ENVP constructs blocked eGFP expression, consistent with our hypothesis. Furthermore, mutation of two hypothetical active sites in the *Pp*ENVP M12 mutant constructs rescued eGFP expression in co-transfection experiments, providing further evidence that the *Pp*ENVP controls a cell death phenotype. These active sites appear to act synergistically as mutation of only one or the other had resulted in minimal eGFP expression rescue. For further investigation of *Pp*ENVP mechanism, it is important to
demonstrate if $Pp$ENVP is required to get into cells to induce cell death. We speculate that $Pp$ENVP functions in a similar way as some well-known endonucleases, which induce conditional suicide intracellularly \(^{47,48}\). However, more laboratory trials are needed to confirm our assumption.

Due to the unclarity of $Pp$ENVP mechanism, there are some challenges to develop $Pp$ENVP into an insecticidal protein. On one hand, $Pp$ENVP should be expressed in genetically modified crops or insect pathogens to achieve oral or infective delivery into pests since arthropod venom proteins rarely possess contact-killing effects \(^{49}\). However, our work suggested that it seems improbable to overexpress $Pp$ENVP in vitro. On the other hand, The range of the cytotoxic activity of $Pp$ENVP across insects must be fully characterized first to determine whether off-target effects in beneficial insects would reduce the overall usefulness of $Pp$ENVP in field applications. We found evidence of cell death in non-natural hosts of $P. puparum$, such as Hi-5 and SF-9 cell lines that originated from the cabbage looper $T. ni$ and the fall armyworm $S. frugiperda$, respectively. These are not the natural hosts of $P. puparum$ but do belong to the same order (Order Lepidoptera) as its natural hosts. Furthermore, we showed that $Pp$ENVP expression constructs injected into $D. melanogaster$ (Order Diptera) embryos also resulted in near-total lethality. $Pp$ENVP appears to have broad-spectrum cell death-inducing activity, but its effects on more insects should be further investigated.

As RNAi knockdown of $Pp$ENVP is not sufficient to rescue the cell death caused by $P. puparum$ venom, our research suggested that several distinct $P. puparum$ venom fractions may have cytotoxic activity. In other words, there are multiple cytotoxic proteins in $P. puparum$ venom. Besides endonucleases, several other kinds of venom proteins have been shown to have cytotoxic activity in parasitoids, including venom phenoloxidase, calreticulin, laccase, and gamma-glutamyl transpeptidase-like protein \(^{50,51}\). Cell death is a complex process – several venom proteins might be involved in the venom of any one parasitoid species, and cell death-inducing strategies may vary drastically across parasitoid species as well. It will be interesting to identify the proteins responsible for cytotoxic activity in other $P. puparum$ venom fractions and in other parasitoid wasps.
In this work, we identified a cell death-inducing endonuclease from the venom of the pupal endoparasitoid wasp, *P. puparum*, and identified two of its activation sites. Although it is not the only cytotoxic protein in *P. puparum* venom, its broad-spectrum activity makes it a potential insecticide candidate gene that should continue to be developed in the future. Future studies should identify the host molecules it targets and the mechanism behind its specificity for particular host cell types. Although our results showed that no additional venom factor was required for *Pp*ENVp cytotoxicity *in vitro*, numerous proteins are known to interact with endonucleases *in vivo*, and it would be important to understand any synergistic interactions that *Pp*ENVp has with other venom components. We believe that the *Pp*ENVp-based products would be rapidly developed since the mechanism of *Pp*ENVp was clarified and contribute to more sustainable biocontrol-mediated IPM systems.

**ACKNOWLEDGMENT**

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REFERENCES


## Tables

### Table 1. Survival of *D. melanogaster* embryos injected with *Pp*ENVP versus control (eGFP) constructs.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Embryo status</th>
<th>χ²</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUAST-attB-cGFP</td>
<td>Didn’t hatch</td>
<td>165</td>
<td>35</td>
</tr>
<tr>
<td>pUAST-attB-PpENVP</td>
<td>Hatched</td>
<td>192</td>
<td>8</td>
</tr>
<tr>
<td>pBID-attB-cGFP</td>
<td>Didn’t hatch</td>
<td>99</td>
<td>66</td>
</tr>
<tr>
<td>pBIDattB-PpENVP</td>
<td>Hatched</td>
<td>400</td>
<td>0</td>
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### Table S1. Primers used in our experiments.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5’-3’)</th>
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<tr>
<td><em>Pp</em>ENVP-qPCR-sp</td>
<td>CCAAGAACCGGCGATTTGAT</td>
</tr>
<tr>
<td><em>Pp</em>ENVP-qPCR-ap</td>
<td>CGGCCTAGGTTGACACAAG</td>
</tr>
<tr>
<td><em>Pp</em>18s-sp</td>
<td>CGAGCGATGAAACCGACAG</td>
</tr>
<tr>
<td><em>Pp</em>18s-ap</td>
<td>CGGGGAGGTAGTGACGAA</td>
</tr>
<tr>
<td><em>Pp</em>ENVP-sp</td>
<td>TTTCACAAACAACAGTCACAAATG</td>
</tr>
<tr>
<td><em>Pp</em>ENVP-ap</td>
<td>TCAATTCTCATTTGCCACTTG</td>
</tr>
<tr>
<td><em>Pp</em>ENVP-pET-28a-sp</td>
<td>AGCAATGGGTCGCGGATCCTTTCACAAACAACACGTCACAAATG</td>
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<tr>
<td><em>Pp</em>ENVP-pET-28a-ap</td>
<td>GTGGTAGTGTCGCTTAGCTATTCATTTGCACCTTG</td>
</tr>
<tr>
<td><em>Pp</em>ENVP-pGEX-4T-2-sp</td>
<td>GTTCGCGTGATCTTTCACAAACAACAGTCACAAATG</td>
</tr>
<tr>
<td><em>Pp</em>ENVP-pGEX-4T-2-ap</td>
<td>ATCGCGCCGCTGAGTCATTTCTATTTGCACCTTG</td>
</tr>
<tr>
<td><em>Pp</em>ENVP-pFASTBac-HTB-sp</td>
<td>GATATCCAAACACATTTTCACAAACAACAGTCACAAATG</td>
</tr>
<tr>
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</tr>
<tr>
<td><em>Pp</em>ENVP-pUAST-sp</td>
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<tr>
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<td>eGFP-ppBID-ap</td>
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</tr>
<tr>
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<tr>
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<tr>
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</tr>
<tr>
<td>Gene</td>
<td>Sequence</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------------------------</td>
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</tr>
<tr>
<td>PpENVP-T7-sp</td>
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<td>PpENVP-T7-ap</td>
<td>TAATACGACTCACTATAGGCTACTGCGCGGAGAT</td>
</tr>
</tbody>
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Lowercase letters show the replaced bases
Figure 1. Identification of the cell death-inducing venom fraction in *Pteromalus puparum*. (A) Venom fractions were isolated using anion-exchange chromatography, and protein content of each fraction was measured by absorbance at 280 nm. The red arrow shows the fraction with cytotoxicity. Absorbance at 280 nm and 214 nm were used to present the detected protein content. (B) Venom was further fractionated using size exclusion chromatography, and protein content of each fraction was measured by absorbance at 214 nm. A cell death-inducing fraction (CDIF) was eluted at the 13-minute point, as shown by the red arrow. (C) The CDIF on a SDS-PAGE gel with BSA for comparison. CDIF presents as a single protein band of ~65 kD. (D) Hi-5
cells incubated with PBS and CDIF. The nuclei of the dead cells show green fluorescence using the CellTox™ Green Cytotoxicity Assay.

Figure 2. Characterization of the *PpENVP* sequence and expression pattern.

(A) The amino acid sequence of *PpENVP*. Yellow box, the predicted signal peptide; Green box, the predicted endonuclease domain; Red triangle, the water activation site; Blue triangle, the Mg²⁺ ion binding site. The matched peptide sequences are underlined. (B) A spectrum of the representative peptide sequence.
(STFVYTSAGIATFR) identified from \textit{PpENVP}. (C) \textit{PpENVP} expression levels in different female tissues measured by RT-PCR. One-way ANOVA and Tukey’s multiple comparison test was used to determine the significant difference with different uppercase letters (A, B) ($p < 0.01$). Error bars represent the mean ± standard deviation (SD) from three biological replicates. H = head, T = thorax, C = carcass (abdomen without ovary and venom gland), O = ovary, V = venom gland. (D) \textit{PpENVP} protein levels in different sexes and tissues as detected by Western blot using anti-\textit{PpENVP} antibody. F = adult female, M = adult male. An Actin antibody was used as a control.

\textbf{Figure 3. eGFP expression in co-transfection experiments.} (A) Brightfield and fluorescence images of Hi-5 cells co-transfected with the eGFP vector and one of five other vectors shows that \textit{PpENVP} suppresses GFP expression. (B) Quantification of eGFP fluorescence intensity after co-transfection with different vector constructs. One-way ANOVA and LSD multiple comparison test was used to determine the significant difference with different uppercase (a-c) ($p < 0.05$), and values are shown as mean ± standard deviation from three biological replicates. (C) Western blot analysis of eGFP protein expression in the different co-transfection treatments, using an antibody against the eGFP His tag. Catalase (CAT) and Vehicle (pIEX-1 vector without a gene cloned into it) act as controls. eGFP, enhanced green fluorescent protein; M1, mutant at the water activation site; M2, mutant at the magnesium ion binding site; M12, mutant at both two sites.
Figure 4. The effects of *Pp*ENVP knockdown on venom cytotoxicity. (A) *Pp*ENVP knockdown resulted in a reduced *Pp*ENVP expression level compared to control eGFP knockdown (*p* < 0.01). (B) SDS-PAGE and Western blot analysis both show that *Pp*ENVP RNAi reduces the amount of *Pp*ENVP protein in whole venom. The red arrow indicates the original *Pp*ENVP band. (C) Quantification of fluorescence intensity (a measure of cell death) in 8-hour treated Hi-5 cells. Orange bars: PBS control; Green bars: venom from eGFP RNAi wasps; Blue bars: venom from *Pp*ENVP RNAi wasps. At each venom concentration level, no significant differences were found between eGFP RNAi and *Pp*ENVP RNAi based on One-way ANOVA and Tukey’s multiple comparison test. Lowercase letters (a, b) were used to determine the significant difference (*p* < 0.05). Values were shown as mean ± standard deviation from three biological replicates.
**Figure S1.** Time-course quantification of fluorescence intensity (a measure of cell death) in treated Hi-5 cells. Orange bars, PBS control; Green bars, venom from eGFP RNAi wasps; Blue bars, venom from *Pp*ENVP RNAi wasps. Results of One-way ANOVA and Tukey’s multiple comparison test were displayed using lowercase letters (a, b) to determine the significant difference (*p* < 0.05). Values were shown as mean ± standard deviation from three biological replicates.