A serpin (CvT-serpin15) of teratocytes contributes to microbial-resistance in *Plutella xylostella* during *Cotesia vestalis* parasitism

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**Abstract**

**BACKGROUND:** Parasitic wasps are an important group of entomophagous insects for pest control. As parasitic wasps often lay eggs on or into their associated hosts, parasitoids evolve to utilize several factors including venom, polydnavirus (PDV) to alter host physiology for successful parasitism. Some taxa of endoparasitoids produce teratocytes, which are a type of cell that is released into host insects when wasp eggs hatch. Teratocytes display multifunction in parasitism such as host nutritional exploitation, immune and developmental regulation, by secreting plenty of proteins into host hemocoel.

**RESULTS:** A serpin (CvT-serpin15) secreted by teratocytes was characterized. QPCR results showed the expressional level of CvT-serpin15 was upregulated following bacterial challenges. Enzyme activity experiment indicated the recombinant CvT-serpin15 protein could interfere with the growth of Gram-positive bacteria *Staphylococcus aureus*. The survival rate assay demonstrated CvT-serpin15 increased survival rate of *Plutella xylostella* infected by *S. aureus*.

**CONCLUSION:** CvT-serpin15 secreted by teratocytes would boost the host immune system when pathogens invade host hemocoel during parasitism, and ultimately protect the development of wasp larva from bacterial infection.

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**Keywords:** teratocytes; serine protease inhibitor; antimicrobial activity; parasitoid; survival rate

1 INTRODUCTION

Endoparasitic wasps are parasitic hymenoptera that live in the host before egression, and they gradually consume and eventually kill the host.1 In order to reach maturity within the host, it must overcome its host immune defenses to survive and maintain a physiological environment that is favorable for its own development at the same time.2 In fact, both maternal and embryonic factors are adopted as specialized strategies of host regulation by endoparasitic wasps.3 Maternal factors such as polydnavirus, venom and ovarian proteins are injected into the host hemocoel during oviposition,4 whereas teratocytes (TCs) as a type of specialized cells are dissociated from the embryonic membrane during the egg hatching.5 Once being released into host hemocoel, teratocytes do not divide but the size and ploidy levels often increase.6 These factors often induce complex physiological alterations in hosts that benefit development of the parasitoid.7

As an important parasitic factor, teratocytes are described to have effects on host nutritional suitability, endocrine balance and immune response.8,9 From nutrition regulation, teratocytes could damage the host tissue and release nutrients into hemocoel,10–13 as well as being fed directly by wasp larva for parasitoid larval development.14 As for endocrine balance, *Microplitis croceipes* teratocytes inhibit the synthesis of juvenile hormone esterase (JHE), ecysteroids titer15 and the extracts of teratocytes act as juvenile hormone in some cases.16 The role of teratocytes in host immune regulations has two sides.8,17,18 On one hand, teratocytes are demonstrated to suppress the host immune capacity for successful parasitization.19–21 Specifically, teratocytes of *Apan teles kariyai* release a substance that inhibited phenoloxidase
(an enzyme that plays a key role in the melanization processes) activity of the host during the late stages of parasitization.\textsuperscript{22} It had been reported that teratocytes from \textit{Meteorus gyrator} (Thunberg) led to reduced phenoloxidase activity and developmental arrest of its noctuid host.\textsuperscript{23} A TIL-type serine protease inhibitor from \textit{Cotesia vestalis} teratocytes was reported to regulate host melanization by inhibiting the activity of \textit{Plutella xylostella} PAP.\textsuperscript{24} On the other hand, several studies have found that teratocytes can produce antimicrobial peptides to compensate the immune response of host.\textsuperscript{18} Previous studies found that teratocytes of \textit{Apanteles glomeratus} could produce fungistatic material to compensate the parasitically induced weakness of host cuticle to fungus infections.\textsuperscript{25} Recent studies also showed that teratocytes could secrete antimicrobial peptides and demonstrated that teratocytes could not only depress host immunity but also produced antimicrobial peptides with functions that helped protect the host from infection by invading pathogens.\textsuperscript{17,18}

Serpins are a superfamily of proteins folding into a conserved tertiary structure with a reactive center loop (RCL) near the C-terminus, which acted as bait for a target protease, which was known to be central in controlling many important proteolytic cascades.\textsuperscript{26} Insect-derived serpins had been identified in \textit{Drosophila melanogaster}, \textit{Manduca sexta}, \textit{Bombby mori}, \textit{Anopheles gambiae} and \textit{Tenebrio molitor}, regulating the activation of melanization and the Toll signaling pathway which controls the synthesis of antimicrobial peptides.\textsuperscript{27–32} The first parasitoid serpin possibly used as a virulence factor that disrupts the PPO activation pathway either by inhibiting PAPs or serine proteases in the activation cascade was identified in \textit{D. melanogaster-Leptopilina bouardi} parasitic system.\textsuperscript{33} It had been widely reported that serpins are involved in humoral immunity. Besides, there are reports of serpins from species of crustacean and hymenoptera insects showing antimicrobial activity, indicating that they may function as direct effectors interacting with pathogens.\textsuperscript{34,35}

In this study, we used \textit{P. xylostella-C. vestalis} as the host-parasitoid system to explore the molecular mechanism of host immune regulation by parasitoid. CvT-serpin15 was cloned from \textit{C. vestalis} teratocytes which contained the conserved domain of serpin and was secreted into host hemolymph by teratocytes. Our results showed that CvT-serpin15 was not involved in host PO cascade regulation, but it had antimicrobial activity and played a role in increasing the survival rate of parasitized \textit{P. xylostella} when infected by Gram-positive bacteria. Thus, we predicted that teratocytes can protect its host from invading pathogens by producing serpin, which was a protein distinct from the antimicrobial peptides.

2 MATERIALS AND METHODS

2.1 Insect collection, rearing and parasitization

Pupae and larvae of \textit{P. xylostella} parasitized by the endoparasitoid \textit{C. vestalis} were initially collected from cabbage fields in the suburbs of Hangzhou, Zhejiang province, China. \textit{P. xylostella} and its endoparasitoid, \textit{C. vestalis} were reared as previously described.\textsuperscript{36} Once emerged, both \textit{P. xylostella} and \textit{C. vestalis} were raised on cabbage grown at 25 °C, 65% relative humidity, and 14 h light: 10 h dark. Adult wasps were fed with 10% (v/v) honey solution. The third instar larvae of \textit{P. xylostella} were parasitized by \textit{C. vestalis} female adults at a 2:1 (host: wasp) ratio for 1 h. The parasitized larvae were allowed to feed on cabbage until formation of cocoons, which were collected in plastic jars for the next cycles. At fixed time intervals after parasitization, the parasitized larvae were dissected to collect teratocytes.

2.2 Collection of teratocytes and venom glands

Teratocytes were collected by the methods described by Wang et al. (2018).\textsuperscript{36} Parasitized \textit{P. xylostella} larvae were surface disinfected in 75% ethanol, dried in the air and then dissected in Serum Free Medium (Thermo Fisher Scientific, USA). Teratocytes and hemocytes were dispersed into medium from hemocoel. After resting for 30 min, teratocytes remained suspended in medium while host hemocytes were attached to the bottom of the culture well. Therefore, teratocytes could be transferred to a micro-centrifuge tube using a pipette with scarcely any hemocyte contamination. For culturing teratocytes \textit{in vitro}, teratocytes were washed at least five times with fresh medium by gently centrifuging the cells at 500 g for 5 min and the supernatant were decanted. Teratocytes were resuspended in fresh medium.

To prepare qPCR samples, teratocytes were dissociated from \textit{C. vestalis} embryo about 48 h post-oviposition at 24 °C, so teratocytes collected at 60 h after parasitization were designated as 1-day old teratocytes in our experiments. Based on this, 1-, 2-, 3-, 4- and 5-day old teratocytes were collected according to the above method. Venom glands (Vg) were collected according to the methods provided by Zhao et al. (2017).\textsuperscript{37} Vgs were dissected from 3-day-old mated female wasps, collected and directly put into Trizol (Vazyme, China), then stored at −80 °C.

2.3 Collection of \textit{P. xylostella} larvae hemolymph and teratocytes culture medium

To confirm whether CvT-serpin15 was secreted into \textit{P. xylostella} hemocoel, the hemolymph was collected as described in Gu et al. (2019).\textsuperscript{24} Paralyzed \textit{P. xylostella} larvae when teratocytes were 1-, 2-, 3-, 4- and 5-day old were surface sterilized with 75% ethanol, washed in sterilized distilled water and air-dried. By cutting the abdominal prolegs of \textit{P. xylostella} larvae on ice using forceps, hemolymph was collected and swiftly transferred into chilled sterile tube using capillaries. Then the hemolymph was diluted by twofold with anticoagulant buffer (4 mM sodium chloride, 40 mM potassium chloride, 8 mM EDTA, 9.5 mM citric acid, 27 mM sodium citrate, 5% sucrose, pH 6.8). The collected hemolymph was centrifuged at 5500 rpm for 10 min at 4 °C. The supernatant, that was cell-free hemolymph was collected and used for immune-blot analysis. The hemolymph of non-parasitized \textit{P. xylostella} larvae of the same instars as the parasitized were used as negative control.

Teratocytes were collected from about 300 parasitized \textit{P. xylostella} larvae at 5 days after parasitization as described in Materials and Methods 2.2. Teratocytes were cultured as previously described.\textsuperscript{38} Teratocytes were transferred into 96-well plates containing 50 μL serum-free medium (Thermo Scientific HyClone, USA) with a final concentration of 10% penicillin-streptomycin solution (Thermo Fisher Scientific, USA) and incubated for 36 h at room temperature. The teratocytes were removed by centrifuging at 650 g for 5 min at 4 °C and the supernatant, TCM containing teratocytes secretory proteins (TSPs) was used for immune-blot analysis.

2.4 RNA extraction and gene cloning

Total RNA was isolated from collected teratocytes using High Pure RNA Isolation kit (Roche, Germany) following the manufacturer’s instructions. The RNA concentration was quantified using a Nanodrop spectrometer 1000 (NanoDrop Technologies, Rockland). The full-length sequence of CvT-serpin15 was gained using the SMART RACE CDNA Amplification kit (Clotech, USA) according to the manufacturer’s instructions. The amplified PCR products were inserted into a pMD-19 T vector (Takara, Japan), verified by specific primers
The CvT-serpin15 protein antisera was prepared by HuaBio Inc. (Hangzhou, China). New Zealand white rabbits were immunized three times for 2-weeks with 0.5 mg purified CvT-serpin15 protein homogenized in complete Freund’s adjuvant. A week later, a booster injection was conducted. The rabbit antisera was collected and purified 7 days after the final immunization and stored at −80 °C.

2.8 Western blot

Each protein sample, i.e. teratocytes culture medium, cell-free hemolymph of parasitized and non-parasitized *P. xylostella* was mixed with SDS protein loading buffer (Sangon, China) and boiled in water for 10 min. Afterwards, the protein samples were separated by 12% SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA) using a Trans-Blot SD Cell and System (Bio-Rad, USA) at 16 V for 20 min. The PVDF membrane was blocked with 3% BSA overnight and washed in 1 × TBST buffer. The CvT-serpin15 in protein samples were detected with CvT-serpin15 polyclonal antibodies (1: 200) as primary antibodies and the β-actin as an internal standard was detected with β-actin mouse monoclonal antibody (1: 2000) (Abcam, UK) followed with anti-rabbit/mouse secondary antibodies conjugated with IgG-horseradish peroxidase (HRP) (Abclonal Tech., China). Protein bands on PVDF membranes were detected using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, USA) and photographed by ChemiDoc MP Imaging System (Bio-Rad, USA).

2.9 Inhibitory assay of rCvT-serpin15

To detect activities of rCvT-serpin15 against serine proteases, the inhibitory activities of the rCvT-serpin15 against five serine proteases, containing trypsin (bovine pancreas, Sigma), α-chymotrypsin (bovine pancreas, Sigma), elastase (porcine pancreas, Sigma), thrombin (human plasma, Sigma) and subtilisin A (protease from *Bacillus licheniformis*, Sigma) were performed. This inhibitory assay was conducted using the method described previously with small modifications. Increasing concentration (0 nM, 0.16 nM, 0.32 nM, 0.48 nM, 0.64 nM, 0.80 nM and 0.96 nM) of rCvT-serpin15 were incubated with 0.43 nM trypsin, 0.4 nM α-chymotrypsin, 0.3 nM thrombin, 0.36 nM subtilisin A and 0.38 nM elastase, respectively for 10 min at room temperature with the total volume adjusted to 50 μL with Tris–HCl buffer (0.1 M Tris–HCl containing 0.1 M NaCl and 1 mM CaCl₂, pH 8.0). The residual protease activities were determined by adding specific chromogenic substrates (1 mM, in 50 mM Tris–HCl buffer containing 50 mM NaCl and 5 mM CaCl₂, pH 7.5), that were Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride (Sigma B3133) for trypsin, N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (Sigma S7388) for α-chymotrypsin, N-Succinyl-Ala-Ala-Pro-Leu p-nitroanilide (Sigma S8511) for elastase, N-(p-Tosyl)-Gly-Pro-Arg p-nitroanilide acetate salt (Sigma T1637) for thrombin and Z-Gly-Gly-Leu p-nitroanilide (Sigma C3022) for subtilisin A, into the reaction mixture. Monitoring OD value at 405 nm with a microplate reader (Thermo Fisher Scientific, USA). One unit of amidase activity was defined as 0.001 ΔA₄₀₀ min⁻¹.

2.10 Immune challenge of teratocytes in vitro and in vivo

Teratocytes were challenged with *E. coli* and *Staphylococcus aureus* to determine if CvT-serpin15 from teratocytes involved in immune response. For induction of teratocytes in vitro, 3-day old teratocytes collected from 40 parasitized *P. xylostella* larvae were cultured in 96-well plates (Thermo Fisher Scientific, USA) with the addition of 2 μL heat-treated *E. coli* (ATCC69925) (OD₆₀₀ = 0.2) or *S. aureus* (ATCC2592) (OD₆₀₀ = 0.2) obtained from...
American Type Culture Collection (ATCC) and sterile TBS as a control. Teratocytes and bacterium were co-cultured and teratocytes were collected at 0 h, 6 h, 12 h, 24 h, 48 h after addition of bacteria. The teratocyte samples challenged or non-challenged were collected for qPCR analysis.

Forty *P. xylostella* larvae at 5 days after parasitization were treated with 75% ethanol for surface sterilization and then injected with heat-treated *E. coli* (1 × 10^6 cells), *S. aureus* (1 × 10^6 cells) or sterile TBS (negative control). *P. xylostella* larvae were dissected at 0 h, 6 h, 12 h, 24 h, 48 h after injection and teratocytes in vivo were collected as the method above mentioned. The teratocyte samples were collected for qPCR analysis.

**2.11 Melanization assays**

To test if CvT-serpin15 affects the immune response of melanization, assays for the inhibitory activities of rcVT-serpin15 on PPO activation and PO activity were conducted according to the methods described by Gu et al. (2019). For inhibition of PPO activation, Each 2 μL of prepared cell-free hemolymph was incubated with 10 μL TBS, 10 μL TBS/Micrococcus luteus (0.5 μg) (Sigma, USA) mixture, 10 μL TBS/M. luteus (0.5 μg)/rcVT-serpin15 (1 μg) mixture, 10 μL TBS/M. luteus (0.5 μg)/BSA (1 μg) as negative control and 10 μL TBS/M. luteus (0.5 μg)/PTU (saturated) as positive control, respectively, for 20 min at 25 °C. 200 μL L-Dopamine substrate (2 mM) was added to the above mixture and OD_{490} was measured in a plate reader (Thermo Fisher Scientific, USA). One unit activity of PO activity was defined as an increase of absorbance at 490 nm by 0.001 per minute (0.001 ΔA_{490} min⁻¹).

For inhibition of PO activity, each 2 μL of prepared cell-free hemolymph was incubated with 10 μL TBS/M. luteus (0.5 μg) mixture. After induction for 45 min, the mixtures were incubated with 10 μL TBS, 10 μL rcVT-serpin15, 10 μL TBS buffer (0.1 M Tris–HCl, pH 6.5), 10 μL bovine serum albumin (BSA, 1 μg mL⁻¹) (Roche, USA) as negative control, 10 μL saturated phenylthiourea (PTU) (Sigma, USA) as positive control for 10 min, respectively. OD_{490} were monitored after adding 200 μL L-Dopamine substrate (2 mM) to the mixtures. PO activity was calculated with the aforementioned method.

**2.12 Growth curve analysis**

The influence of CvT-serpin15 on the growth of microbes were tested following the procedure described by Wang et al. (2013). *S. aureus* and *E. coli* (New England Biolabs, USA) were grown to log-phase at 37 °C in Mueller Hinton (MH) broth. Cultures were then serially diluted with fresh MH broth to an OD_{600} = 0.006. 80 μL diluted cultures were incubated with 20 μL rcVT-serpin15 (3.5 mg mL⁻¹) or TBS buffer (0.01 mM Tris–HCl, pH 7.2) or Ampicillin (1 mg mL⁻¹). The bacterial concentration was monitored by measuring OD_{600} with a plate reader (Thermo Fisher Scientific, USA) every other 5 min from 0 min to 400 min. Then growth curves were plotted based on the differences of OD_{600} values obtained.

**2.13 Mortality assay**

Fifty third-instar non-parasitized *P. xylostella* larvae were injected with 0.025 μL rcVT-serpin15 (0.4 μg mL⁻¹) plus 0.025 μL *S. aureus* (OD_{600} = 0.05) or *E. coli* (OD_{600} = 0.05) per larva, 0.025 μL TBS plus 0.025 μL *S. aureus* (OD_{600} = 0.05) or *E. coli* (OD_{600} = 0.05) as a positive control and 0.05 μL TBS as a negative control by FemtotJet® Microinjection instrument (Eppendorf, Germany). The survival larvae were counted at 12 h, 24 h, 36 h after injection, respectively. Every treatment was repeated three times.

**2.14 Statistical analysis**

All data were calculated as mean ± S.E. One-way analysis of variance (ANOVA) with Turkey-test was used to determine significant differences between samples. Significance threshold was set as *P* < 0.05.

**3 RESULTS**

**3.1 Characterization and phylogenetic tree of CvT-serpin15**

A 1359 bp sequence fragment (unigene1762), obtained from assembled *C. vestalis* teratocytes transcriptome (Accession number: SRR531389), encoded a protein fragment which was annotated as a serpin. The missing ends of the transcripts were obtained by RACE. The *Cvt-serpin15* gene was located at scaffold29_104 of *C. vestalis* genome containing five introns and six exons (Fig. 1(A)). The full-length sequence of *Cvt-serpin15* (GenBank accession number: APD76157.1) was 1405 bp (base pairs) with an open reading frame of 1239 bp encoding 412-amino acid (aa) protein. A SERPIN (serine protein inhibitor) domain (position 45–409 aa) was predicted by SMART (simple modular architecture research tool) server (E-value = 3.85e-117). A signal peptide sequence of 25 aa was determined by SignalP4.1 server (http://www.cbs.dtu.dk/services/SignalP/) at the N-terminal portion of the deduced amino acids (Fig. 1(A)). The protein encoded by CvT-serpin15 has a theoretical molecular weight of 43.6 kDa and an isoelectric point of 5.23. Multiple alignments suggested that CvT-serpin15 had similar serpin conservative motifs and serpin signature with that from other insects. The predicted P1-P1’ cleavage site was Met^{270}-Ser^{271} (Fig. 1(B)). The phylogenetic analysis indicated that CvT-serpin15 was grouped to the same cluster with leukocyte elastase inhibitor-like (Fa-LEI-like) of *Fopius artisans*, which belongs to the serpin superfamily of hymenoptera (Fig. 1(C)).

We identified the transcripts of *Cvt-serpin15* in transcriptome of both venom gland and teratocytes (Gao et al., 2016; Zhao et al., 2017). The transcripts of *Cvt-serpin15* were detected in venom gland and *C. vestalis* teratocytes. The results showed that the transcriptional level of *Cvt-serpin15* in venom gland was one tenth of the transcriptional level in teratocytes (Fig. 1(D)). The transcriptional level of *Cvt-serpin15* in 3-day old teratocytes was the highest (Fig. 1(D)).

**3.2 Inhibitory activity of rcVT-serpin15 on serine proteases**

The N-terminal 6 × His-tagged rcVT-serpin15 protein with molecular weight of about 42 kDa was purified (Supporting Information, Figure S1). Total 4.5 mg rcVT-serpin15 was obtained from 2-L *E. coli* culture. Our results showed that rcVT-serpin15 could inhibit activities of trypsin (Fig. 2(A)), α-chymotrypsin (Fig. 2(B)), elastase (Fig. 2(C)) and subtilisin A (Fig. 2(E)) by 40.45%, 97.25%, 94.14% and 93.76%, respectively, when the molar ratio of rcVT-serpin15/trypsin, rcVT-serpin15/α-chymotrypsin, rcVT-serpin15/elastase and rcVT-serpin15/subtilisin A reached 2.2, 2.3, 4.4, 2.6, but rcVT-serpin15 had no inhibitory activity on thrombin (Fig. 2(D)).

**3.3 Secretion of CvT-serpin15 by teratocytes**

When teratocytes were culture in vitro, they would secret many proteins into medium. The deduced amino acid sequence of *Cvt-serpin15* contained a signal peptide, indicating CvT-serpin15 might be secreted by teratocytes. We detected an obvious protein band in teratocytes culture medium by using rabbit anti-rcVT-
serpin15 polyclonal antibody (Fig. 3(A)), suggesting that *C. vestalis* teratocytes secreted CvT-serpin15 into extracellular space. Further, our results also showed that parasitized host plasma with 1–5-day-old teratocytes had immune reactions with CvT-serpin15 polyclonal antibody, but not non-parasitized plasma of *P. xylostella* (Fig. 3(B)). Meanwhile, the number of CvT-serpin15 bands in *P. xylostella* plasma was the most abundant when teratocytes were 3-day old and the immunoreactive bands were weaker when teratocytes were 1-, 4- and 5-days old (Fig. 3(B)). ⊎-actin band was included for equitable loading. Therefore, CvT-serpin15 protein was secreted by *C. vestalis* teratocytes into *P. xylostella* hemolymph and involved in regulating host inner physiological condition.

### 3.4 Increasing transcriptional level of CvT-serpin15 in response to bacterial challenge

Most serpins verified in insects are involved in immune response.46 We obtained expresional pattern of CvT-serpin15 in teratocytes in vivo or in vitro after challenges with killed *E. coli* (G−) and *S. aureus* (G+). QPCR assays indicated that transcriptional level of CvT-serpin15 in teratocytes in vivo was up-regulated at 48 h after challenge by *S. aureus* (Fig. 4(D)). Transcriptional level of CvT-serpin15 in teratocytes in vitro was up-regulated from 6 h after challenge by *S. aureus* and reached the highest value at 48 h (Fig. 4(B)). However, CvT-serpin15 transcriptional level in teratocytes in vitro was almost not affected after challenge by *E. coli* (Fig. 4(A)). CvT-serpin15 transcriptional level in teratocytes in vivo also was up-regulated at 48 h after challenge by *E. coli* (Fig. 4(C)).

### 3.5 No effect on melanization

In order to investigate the function of CvT-serpin15, we tested the effect of rCvT-serpin15 on melanization. The *P. xylostella* hemolymph has low basal PO activity and could be activated dramatically by bacteria.30 Cell-free hemolymph was first activated by *M. luteus* and then was incubated with rCvT-serpin15 to test
whether CvT-serpin15 inhibits the activity of PO itself. The results showed that the PO activity of cell-free hemolymph was not inhibited by rCvT-serpin15 (Fig. 5(A)). Also, cell-free hemolymph was incubated with rCvT-serpin15 firstly and then M. luteus was added to test whether CvT-serpin15 is involved in the process of PPO activation. The results suggested the PPO activation was also not inhibited by rCvT-serpin15 (Fig. 5(B)).

3.6 Inhibition on the growth of Gram-positive bacteria

Serpin was reported to inhibit the growth of bacteria.47–51 The inhibitory effect of the rCvT-serpin15 on the growth of bacteria was examined to determine its antimicrobial activity. The Gram-positive bacterium S. aureus and Gram-negative bacterium E. coli were tested. The results suggested that there was no significant growth difference of E. coli treated with rCvT-serpin15 and the negative control (Fig. 6(A)). However, the results indicated that the growth of S. aureus was significantly suppressed by rCvT-serpin15 from 200 min post-treatment in comparison to the negative control (Fig. 6(B)).

3.7 Increasing survival rate of bacteria-infected host larvae

To investigate the antimicrobial activity of CvT-serpin15 in vivo, rCvT-serpin15 was injected into non-parasitized P. xylostella larvae together with S. aureus, which was used as invading pathogenic bacteria. The survival rate was 100% in the negative control group injected with just TBS. The results suggested that the group injected with rCvT-serpin15 had a higher survival rate than the positive control group when P. xylostella larvae were infected with S. aureus, with an increase survival rate of 27% at 36 h post infection (Fig. 7(B)). However, the results also showed that there was no significant difference of survival rate between the group injected with rCvT-serpin15 and the positive control group when P. xylostella larvae were infected with E. coli (Fig. 7(A)).

4 DISCUSSION

Serine proteinase inhibitors, widely distributed in organisms, play key roles in a variety of biochemical pathways including hemolymph coagulation, PPO activation and antimicrobial synthesis. To date, many studies of serpins have been published in insects including B. mori,52–54 M. sexta,30,31,55–59 Mythimnan unipuncta,60 A. gambiae,61,62 D. melanogaste,63 T. molitor27,29 and O. furnacalis.64 Serpin-derived from hymenopteran parasitoid has not been extensively studied. The first reported serpin in parasitic hymenoptera was identified from the parasitoid wasp L. boulardi, which suggests serpins might act as virulence factors among parasitoids.33 In this study, a serpin gene named CvT-serpin15 was cloned and characterized from C. vestalis teratocytes. A conserved SERPIN region existed in the amino acid sequence of CvT-serpin15, similar to other previously reported serpins.32 The predicted P1-P1' cleavage site was Met-Ser, which was the same as the Fa-LEI-like protein. Our results further proved that CvT-serpin15 was secreted by C. vestalis teratocytes into the hemocoel of host P. xylostella (Fig. 3(A) and (B)).

Figure 3. CvT-serpin15 was secreted by Cotesia vestalis teratocyte into Plutella xylostella hemolymph. (A) Detection of CvT-serpin15 in TCM. SDS-PAGE of TCM was followed by immunoblotting using antibody against rCvT-serpin15. Lane: M, protein marker; TCM, teratocytes culture medium; CK, blank culture medium. (B) Detection of CvT-serpin15 in host hemolymph parasitized by C. vestalis. β-actin was used as an internal standard. NP, non-parasitized host larvae by C. vestalis; P, parasitized host larvae by C. vestalis.

Figure 2. Inhibitory effect of rCvT-serpin15 on the activities of commercial serine proteases. (A), (B), (C), (D) and (E) represent inhibitory effect on trypsin, α-chymotrypsin, elastase, thrombin and subtilisin A, respectively. The inhibitory effect of rCvT-serpin15 was plotted as the residual activity of proteases against the molar ratio of rCvT-serpin15/proteases. Error bars represent the mean ± S. E. (N = 3).
Figure 4. Expression pattern of CvT-serpin15 in teratocytes in vitro and in vivo after microbial challenge. Teratocytes collected from 5 days Plutella xylostella larvae parasitized by Cotesia vestalis were induced with inactivated E. coli (A), Staphylococcus aureus (B) in vitro for 0 h, 6 h, 12 h, 24 h and 48 h. Five days P. xylostella larvae after parasitism were challenged with E. coli (C), S. aureus (D), then teratocytes in vivo were collected. Error bars represent mean $\pm$ S. E. (N = 3). Different letters above the bars are significantly different (one-way ANOVA followed by Tukey-test, $P < 0.05$).

Figure 5. Inhibition of activated PO itself (a) and PPO activation (b) of Plutella xylostella hemolymph by rCvT-serpin15. TBS, Blank; BSA, negative control; PTU, positive control; inactivated M. luteus is an elicitor. Error bars represent the mean $\pm$ S. E. (N = 3). The data were conducted by one-way analysis of variance (one-way ANOVA, Tukey-test, $P < 0.05$). Significant differences were indicated with different letters.
extracellular space. qPCR results showed that CvT-serpin15 was transcribed in venom gland, but the transcriptional level was much lower. It is reported that the venom from P. hypochondriaca contains several enzymes including protease inhibitors, neurotoxin-like factors and anti-hemocyte aggregation compounds, which could be involved in venom homeostasis. Though the role of CvT-serpin15 in venom gland was not investigated in this study, our results showed that rCvT-serpin15 could inhibit the activities of several serine proteases. We speculated that the function of CvT-serpin15 in venom reservoir might be a protease inhibitor to inhibit or stabilize venom enzymes for keeping venom homeostasis. As the parasitoid larva develops within the host body, it must overcome its immune defenses to survive, and at the same time maintain an environment that is supportive to its own development to reach maturity. Parasitic factors (PDV, venom and teratocytes) combined to depress host immune system for successful parasitization. Studies also suggested C. vestalis teratocytes synthesize and secrete immune-depressive factor(s). Kitano et al. (1990) showed that teratocytes of C. glomerata inhibit the phenoloxidase activity of the host, Pieris rapae. Meanwhile, most serpins identified in insects inhibit serine proteases to block PO cascade. Since the expressional level of CvT-serpin15 was up-regulated after bacteria challenge in both in vivo and in vitro teratocytes (Fig. 4(B) and (D)), we suspected that CvT-serpin15 secreted by C. vestalis teratocytes might be an immune-depressive factor to suppress immune response of P. xylostella melanization. Surprisingly, our results demonstrated that CvT-serpin15 did not affect the PPO activation and inhibit the activity of PO itself. The result of the growth of S. aureus was suppressed by rCvT-serpin15 in our study was similar to many other serpins, which had been shown to affect the growth of certain bacteria. For instance, Lvserpin inhibited the growth of a Gram-negative bacteria, V. anguillarum. Serine protease inhibitor from bumblebee.
venom was shown to bind directly to Bacillus subtilis, B. thuringiensis and Beauveria bassiana but not to E. coli. Similarly, AcSecapin-1 from bee venom could also bind to bacterial and fungal surfaces and exhibited antimicrobial activity against fungi and Gram-positive and Gram-negative bacteria. Esserpin and RoOserpin affected the growth of E. coli to some extent. RanaSerpin from frog (Rana grahami) eggs could suppress the growth of Gram-positive B. subtilis. A serpin in the kuruma shrimp Marsupenaeus japonicus had antibacterial activity but no inhibitory effect on the PPO activation. The mechanism underlying serpins suppressing bacterial growth has not been deciphered, we speculated that it may affect their growth of bacteria by inhibiting proteolytic activity of the secreted proteinase from bacteria. Previous study showed that Ranaserpin could inhibit the growth of B. subtilis by inhibiting the activity of trypsin, elastase and substilisin produced by the bacteria. The secreted proteinases of the pathogenic microorganism are key components of invasive cocktails and required for entry into the host and rapid utilization of its constituent protein. Nevertheless, the mechanism of bacterial inhibition of CvT-serpin15 needs further study. CvT-serpin15 protein was secreted into P. xylostella hemoloc to exert its function inside P. xylostella. After injection of rCvT-serpin15 into P. xylostella larvae, the survival rate of the larvae was higher than the control group after infection by S. aureus (Fig. 7(B)). From the above results, we inferred that, in order to create a beneficial microenvironment for the development of wasp larvae, CvT-serpin15 from teratocytes might protect the host from invasion of pathogenic microorganisms by interfering with the protease activity they secreted.

In summary, a novel serpin from C. vestalis teratocytes was cloned and characterized. The CvT-serpin15 could not depress the host P. xylostella prophenoloxidase (proPO)-activating system. However, our work provides a novel view of the antimicrobial functions of a parasitoid wasp serine protease inhibitor. CvT-serpin15 could improve survival rate of P. xylostella larvae infected with S. aureus. Previous studies indicated that parasitic factors injected into host (PDVs, venom) at oviposition or produced during the wasp development (teratocytes) could disable host immune response, which leads to a higher probability of invasion of parasitized hosts by pathogenic microorganisms. Previous studies also indicated teratocytes produced antimicrobial peptides to protect the host from infection by other microorganism and suggested teratocytes had bi-directional control function. Together, we concluded CvT-serpin15 from the parasitoid wasp C. vestalis teratocytes might function as the antimicrobial peptide to compensate the weakened immunity of parasitized P. xylostella larvae through a different way.

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SUPPORTING INFORMATION
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