Role of a Serine Protease 27-like Gene in the Innate Immunity of Helicoverpa Armigera

Xuemeng Du[1], Xin Zhang[1], Yang Liu[1], Gang Wang[2,*]

[1]School of Bioscience and Technology, Weifang Medical University, Weifang, China;

[2]School of Bioscience and Technology, Weifang Medical University, 7166 Baotong West Road, Weicheng District, Weifang, 261053, China.

*Corresponding author's E-mail: gangw@wfmc.edu.cn

Abstract

Ecdysone hormone not only mediates the development of insects but also plays an important role in regulating their innate immunity. However, the detailed mechanisms of this hormone remain unclear. In the present research, we identified a serine protease 27-like gene named HaSP27 in Helicoverpa armigera. HaSP27 is highly expressed in fat bodies and hemocytes, and HaSP27 protein is mainly located in the cytoplasm of the granulocytes and plasmatocytes of hemocytes. Bacterial challenge significantly upregulated HaSP27 expression. HaSP27 silencing suppressed bacterial clearance in hemocytes and impaired the expression of AMPs in fat bodies. Treatment with 20-hydroxyecdysone (20E) induced HaSP27 expression through its receptors EcR and USP. These results suggest that HaSP27 acts downstream of the 20E signaling pathway and performs essential functions in the immune response of insects. Our study offers important insights into the mechanisms through which steroidal hormone signals mediate the immune response in insects.

Keywords

Serine Protease; Innate Immunity; 20-hydroxyecdysone; Antibacterial Peptides.

1. Introduction

Although they lack an adaptive immune system, insects have evolved an effective innate immune system to defend themselves against invading pathogens. The innate immunity of insects includes cellular and humoral immune responses. The cellular immune response is mainly medicated by granulocytes and plasmatocytes in hemocytes through encapsulation, phagocytosis, and nodulation [1, 2]. The humoral immune response mainly includes the synthesis of antimicrobial peptides (AMPs) and other effector factors, such as lysozyme, lectin, and polyphenoloxidase [3].

The innate immune response of insects represents a complex regulatory network. Recent studies have suggested that the ecdysone hormone 20-hydroxyecdysone (20E), which is analogous to glucocorticoids in mammals, helps regulate the innate immune response [4, 5]. Some studies have shown that 20E not only induces AMPs expression in the Drosophila S2 cell line but also enhances hemolytic phagocytosis through its receptors EcR and USP [6]. In Drosophila malpighian tubules, 20E rapidly induces AMPs expression through the early gene broad complex even without immune challenge [7]. However, some studies demonstrated the opposite results; Tian, for example, found that injection of 20E suppresses AMPs expression during the molting and metamorphosis of Bombyx mori, which suggests that 20E negatively mediates innate immunity [8]. Given these conflicting results, how ecdysone modulates insect innate immunity and the detailed mechanisms involved in this process remain incompletely understood.

In the present study, we identified a serine protease (SP) 27-like gene named *HaSP27* in *Helicoverpa armigera*. HaSP27 belongs to the trypsin-like SP family, which influences a wide range of physiological processes, such as blood coagulation, digestion, fertilization, and the innate immune response [9]. We investigated the functions of the *HaSP27* gene in *H. armigera* and found that this gene is highly expressed in hemocytes and fat bodies. HaSP27 protein is mainly distributed in the

plasmatocytes and granulocytes of hemocytes. Bacterial challenge significantly upregulated the expression of *HaSP27*, and *HaSP27* silencing in vivo not only significantly increased the bacterial load of hemolymphs but also suppressed the AMPs induced by *Escherichia coli* in fat bodies. Treatment with 20E induced the expression of *HaSP27* through its receptors EcR and USP. These results suggest that 20E regulates *HaSP27* and positively mediates innate immunity in *H. armigera*. Our findings provide interesting insights into the mechanisms through which steroidal hormone signaling pathways mediate the immune response in insects.

2. Materials and methods

2.1 Insects

Cotton bollworms were maintained in a constant-temperature incubator at 26 ℃ under a 12 h/12 h light/dark cycle and reared on an artificial diet consisting of wheat and soybean [10].

2.2 Recombinant protein expression and antiserum preparation

According to the full-length sequence of the *HaSP27* gene (GenBank No.LOC110381573), a portion of the open reading frame (729 bp) encoding the trypsin-like SP conservative domain was amplified using the primers SP27F1 (5'-tactcactgcagcattactggtaatactgg-3') and SP27R1 (5'-tactcagaattctaccca ggatctgaagag-3') with PstI and EcoRI restriction sites at both ends of the amplified sequence. The amplified sequence was inserted into the pET-32a vector and transformed into BL21. The transformed *E. coli* were cultured in LB medium and induced by 0.5 nM IPTG (isopropyl-β-D-thiogalactopyranoside). The soluble recombinant peptides were purified and used as antigens for rabbit antiserum preparation according to a previously described method [11].

2.3 Quantitative real-time PCR and immunocytochemistry

The expression of *HaSP27* was analyzed by qRT-PCR. Total RNA was extracted using the RNAprep Pure Tissue Kit (TIANGEN, China), and first-strand cDNA was synthesized via a previously described method [12]. *HaSP27*-specific primers, namely, SP27F2 (5'-gcctgaagaatgacgttgcc-3') and SP27R2 (5'-tcacggcg taacggtcgtgg-3'), were designed according to the trypsin-like SP conserved domain sequence, and qRT-PCR was performed according to a previously described method [13]. Actin-specific primers, actinF (5'-cctggtattgctgaccgttgc-3') and actinR (5'-ctgttggaaggtggagagggaa -3'), which amplified a 150 bp fragment, were used as a quantitative control.

Immunocytochemistry was performed to analyze the distribution of HaSP27 protein in hemocytes according to a previously described method [11].

2.4 Bacterial challenge and hormonal treatment

Bacterial challenge was performed according to a previously described method [14]. Hemocytes and fat bodies were collected 1, 3, 6, 12, and 24 h after bacterial challenge for expression profile analysis by qRT-PCR. Hormonal treatment was performed according to a previous article [12].

2.5 RNA interference (RNAi) and bacterial clearance assay

Double-stranded RNA (dsRNA) was synthesized using the MEGAscript RNAi Kit, and the primers SP27iF (5'-gcgtaatacgactcactataggacgggtcgtacgctggagtc-3') and SP27iR (5'-gcgtaatacgactcactat aggcgaagctcctatgaagctgg -3') were designed for *HaSP27*-specific dsRNA synthesis. The EcR-B1- and USP1-specific primers for dsRNA synthesis were designed according a procedure we previously described [12]. The synthesis of dsRNAs was performed according to the manufacturer's instructions.

Sixth-instar feeding larvae were used for *HaSP27*-specific dsRNA injection, and the cultured hemocytes were used for EcR-B1 and USP1 silencing. After *HaSP27* silencing in vivo, we performed bacterial clearance assays as described in a previous article [14].

3. Results

3.1 HaSP27 was highly expressed in hemocytes and fat bodies during molting and metamorphosis

Total RNA was extracted from six different tissues of fifth-instar feeding larvae to investigate the tissue distribution of HaSP27. Results showed that the transcription levels of *HaSP27* in fat bodies and hemocytes are significantly higher than those in the midgut, hindgut, foregut, and integument (Fig 1A).

Total RNA was extracted at different stages from the fifth instar 12h to sixth instar 120h larvae to elucidate the developmental expression patterns of *HaSP27* in fat bodies and hemocytes. Results showed that the mRNA level of *HaSP27* is low during the feeding stage and increases at the fifth instar 36h and sixth instar 96h and 120h stages as the larvae undergo molting and metamorphosis (Fig 1B).

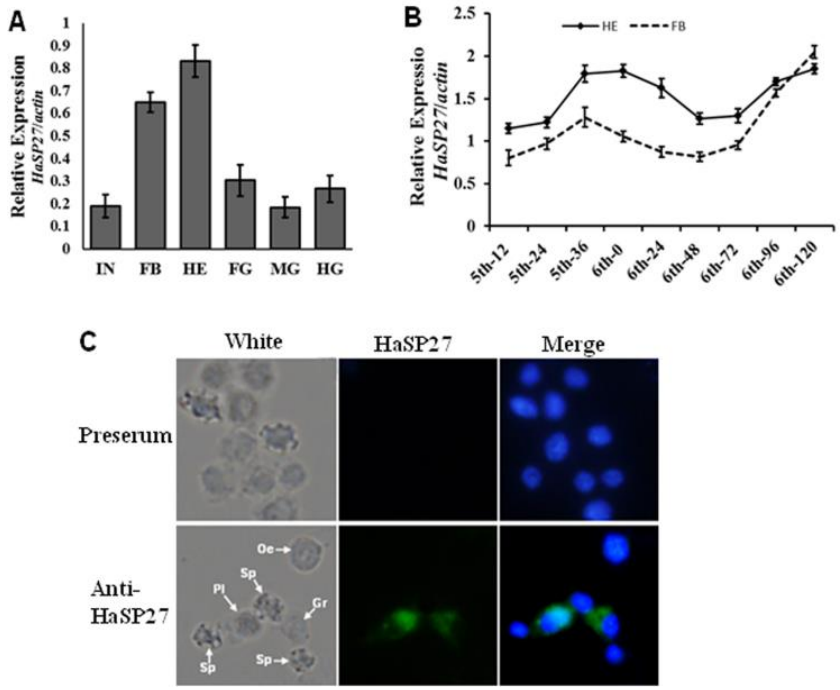

Fig 1. Expression pattern and tissue distribution of *HaSP27*. (A) Real-time PCR results of the expression patterns of *HaSP27* in the IN, FB, HE, FG, MG and HG f fifth-instar feeding larvae. (B) Real-time PCR results of the temporal expression profiles of *HaSP27* in FB and HE of fifth instar 12h to sixth instar 120h larvae. (C) Immunocytochemical results of the distribution of HaSP27 in the hemocytes of fifth-instar molting larvae. Blue portions (DAPI-stained) indicate nuclei. Oe: oenocytoids, Sp: spherulocytes, Gr: granulocytes, Pl: plasmatocytes, Bar = 10 mm. Error bars indicate mean ± SD (n = 3).

3.2 HaSP27 was localized in the granulocytes and plasmatocytes of hemocytes and induced by bacterial challenge

The location of HaSP27 in hemocytes was analyzed by immunocytochemistry. The fluorescence signals of HaSP27 were observed in the cytoplasm of granulocytes and plasmatocytes; spherulocytes, oenocytoids, and prohemocytes did not show these signals (Fig 1C). Considering that granulocytes and plasmatocytes participate in innate immunity, we hypothesize that HaSP27 may be involved in the immune response of insects.

To examine whether HaSP27 is involved in the insect immune response, we performed immune challenge experiments with *E. coli* using sixth-instar feeding larvae and analyzed the expression profiles of *HaSP27* in hemocytes and fat bodies after bacterial challenge. The results showed that

HaSP27 expression levels significantly increased in hemocytes 6, 12, and 24 h after *E. coli* challenge compared with those of the control larvae, which were challenged with PBS; *HaSP27* profiles in fat bodies were similar with those in hemocytes, and the mRNA level of *HaSP27* significantly increased 12 and 24 h after *E. coli* challenge (Fig 2A, B).

3.3 Silencing of HaSP27 in vivo significantly increased the bacterial load in hemolymphs

To investigate the functions of *HaSP27*, we performed RNAi experiments in vivo. Sixth-instar feeding larvae were injected with the dsRNA of *HaSP27*, and the expression level of the gene in hemocytes was analyzed after 48 h by qRT-PCR. The results showed that the expression of *HaSP27* is significantly suppressed by dsRNA compared with the control, which was injected with dsGFP (Fig 2C).

Larvae injected with dsRNA were used for bacterial clearance assays, and results showed that the *E. coli* load was significantly higher in hemolymphs of *HaSP27*-silenced larvae than in the control larvae, which were injected with dsGFP (Fig 2D). These results suggest that *HaSP27* plays an important role in the defense against invading pathogens.

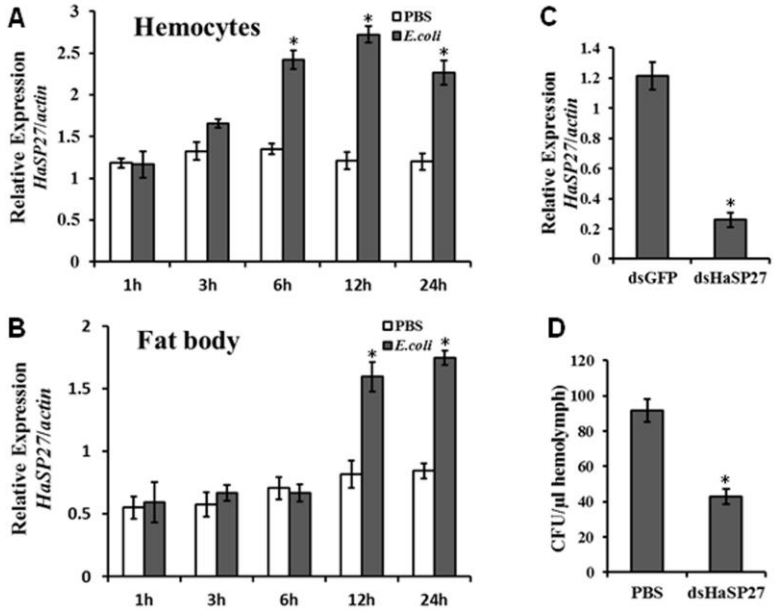

Fig 2. *HaSP27* is involved in the innate immunity of *H. armigera*. (A) Real-time PCR results of the expression profiles of *HaSP27* in hemocytes 1, 3, 6, 12, and 24 h after bacterial challenge. (B) Real-time PCR results of the expression profiles of *HaSP27* in fat bodies 1, 3, 6, 12, and 24 h after bacterial challenge. (C) Real-time PCR results of the effect of RNAi on hemocytes 48 h after dsRNA injection. (D) Bacterial clearance assay results of *HaSP27*-silenced sixth-instar feeding larvae 6 h after *E. coli* challenge; the Y-axis represents the number of colonies (U/μL) in hemolymph. Error bars indicate mean ± SD (n = 3). *p < 0.05 vs. the control.

3.4 Silencing of HaSP27 suppressed the expression of AMPs in fat bodies

To clarify the role of *HaSP27* in the defense against invading pathogens, we analyzed the expression profiles of AMPs genes in *HaSP27*-silenced larvae after bacterial challenge. The results showed that the expression of cecropin-2 is significantly lower 6 and 12 h after *E. coli* challenge in *HaSP27*-silenced larvae than in the control larvae, which were injected with dsGFP (Fig 3A). The expression level of gallerimycin-like was significantly lower 6 h after challenge in *HaSP27*-silenced larvae than in the control larvae (Fig 3C). Attacin and moricin-like showed similar expression profiles, and the expression levels of these genes were significantly lower 24 h after challenge in *HaSP27*-silenced larvae than in the controls (Fig 3B, D). These results suggest that *HaSP27* is involved in the induction of AMPs following the infection of pathogenic microorganisms.

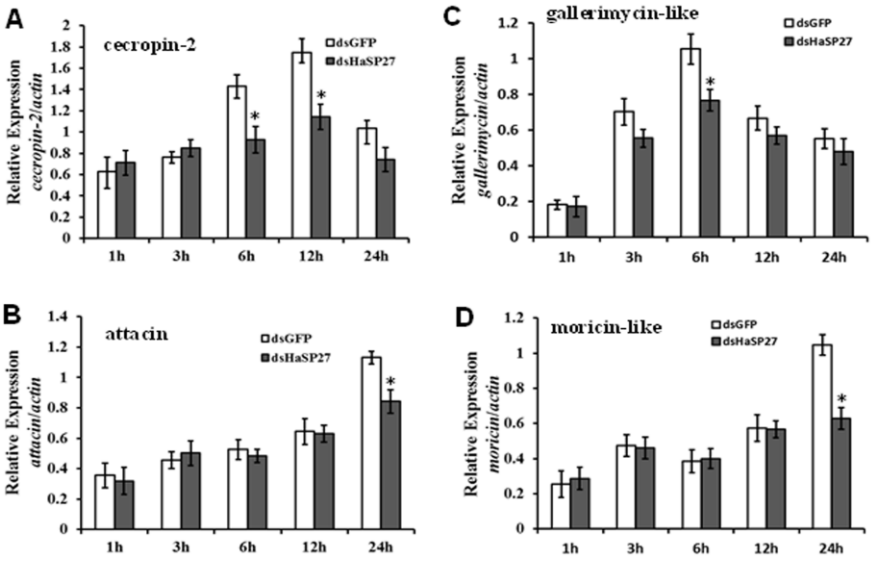

Fig 3. Silencing of *HaSP27* suppresses the expression of AMPs in fat bodies. (A) Real-time PCR results of the expression profiles of cecropin-2 in the fat bodies of *HaSP27*-silenced or control larvae injected with dsGFP 1, 3, 6, 12, and 24 h after *E. coli* challenge. (B) Real-time PCR results of the expression profiles of attacin in the fat bodies. (C) Real-time PCR results of the expression profiles of gallerimycin-like in the fat bodies. (D) Real-time PCR results of the expression profiles of moricin-like in the fat bodies. Error bars indicate mean ± SD (n = 3). *p < 0.05 vs. the control.

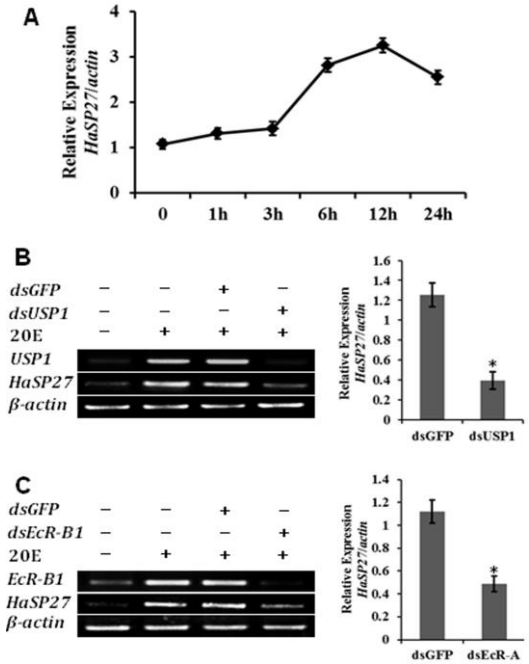

Fig 4. The 20E signaling pathway regulates *HaSP27*. (A) Real-time PCR results of the expression profiles of *HaSP27* in hemocytes of sixth-instar feeding larvae 0, 1, 3, 6, 12, and 24 h after 20E treatment. (B) Semi-quantitative RT-PCR results of the expression of *HaSP27* in hemocytes after USP1 silencing. (C) Semi-quantitative RT-PCR results of the expression of *HaSP27* in hemocytes after EcR-B1 silencing. Error bars indicate mean ± SD (n = 3). *p < 0.05 vs. the control.

3.5 20E upregulated the expression of HaSP27 through EcR and USP

Having observed that *HaSP27* expression increases when larvae undergo molting and metamorphosis and considering that 20E is a key steroidal hormone that regulates insect molting and metamorphosis, we investigated the effects of 20E on *HaSP27* expression. Feeding larvae were injected with 20E,

and the expression profile of *HaSP27* in hemocytes was analyzed by qRT-PCR. The results showed that *HaSP27* expression significantly increases from 6 h to 24 h after 20E treatment (Fig 4A), thus suggesting that the 20E signaling pathway may induce the expression of *HaSP27*.

To determine whether EcR and USP are necessary for *HaSP27* induction by ecdysone, we silenced EcR-B1 and USP1 by RNAi in cultured hemocytes in vitro. Semi-quantitative RT-PCR was used to detect *HaSP27* expression 36 h after dsRNA treatment. The results showed that silencing of EcR or USP leads to the suppressed expression of *HaSP27* (Fig 4B, C). These results suggest that ecdysone induces *HaSP27* expression through its receptors EcR and USP.

4. Discussion

In this study, a gene named *HaSP27*, which belongs to the SP superfamily, was identified in *H. armigera*. *HaSP27* is highly expressed in hemocytes and fat bodies, and HaSP27 protein is mainly distributed in the plasmatocytes and granulocytes of hemocytes. Bacterial challenge significantly induced the expression of *HaSP27*, and silencing of *HaSP27* in vivo not only significantly increased the bacterial load in hemolymphs but also suppressed AMPs induction in fat bodies. 20E can induce the expression of *HaSP27* through its receptors EcR and USP. These results suggest that *HaSP27* is induced by the ecdysone signaling pathway and assists in an insect's ability to protect itself against pathogens.

SPs consist of a large family of proteolytic enzymes. Previous research showed that SPs take part in a wide range of physiological processes, such as immune actions, development, digestion, and fertilization; the human homologous gene TMPRSS13, a transmembrane SP, plays a critical role in colorectal cancer progression [9, 15, 16]. The *HaSP27* of *H. armigera* contains a typical Tryp_SPc domain. Amino acid sequence alignment showed that the Tryp_SPc domain shares only 27% and 28% identity with mouse (NP_001013391.2) and human (NP_001070731.1) homologs (data not shown). This finding suggests that *HaSP27* has very low homology to mammal homologs and that its function may be different from that of mammals. Expression pattern analysis showed that *HaSP27* expression is significantly higher in fat bodies and hemocytes than in the midgut, hindgut, and foregut, which are food-digestive sites. As such, we hypothesize that *HaSP27* plays roles in fat bodies and hemocytes rather than food digestion. The expression profile of the gene obtained at different developmental stages showed that *HaSP27* expression increases during molting and metamorphosis when the larvae undergo integument revival and histolysis. However, we did not detect any defects in larval development after *HaSP27* knockdown. Thus, whether *HaSP27* affects larval development requires further investigation.

20E is a critical steroidal hormone that not only mediates insect molting and metamorphosis but also participates in apoptosis and reproduction [17]. Recent studies have indicated that 20E mediates innate immunity; the 20E signaling pathway may crosstalk with the IMD pathway to mediate AMPs expressions [7], but the detailed mechanism is not clear. Our results revealed that *HaSP27* silencing suppresses the expression of AMPs in fat bodies and that the 20E signaling pathway could upregulate the expression of *HaSP27* through its receptors EcR and USP. Taken together, the results indicate that ecdysone may positively regulate innate immunity by mediating *HaSP27*.

In conclusion, we identified an SP-like gene in *H. armigera* named *HaSP27*. This gene is highly expressed in the fat bodies and hemocytes of larvae, and immune challenge significantly induced *HaSP27* expression. Silencing of *HaSP27* suppressed bacterial clearance in hemocytes and impaired AMPs expressions in fat bodies. The 20E signaling pathway regulates *HaSP27* expression through EcR and USP. The results suggest that HaSP27 acts downstream of the 20E signal pathway and supports the immune response of insects. Our study provides novels insights into the mechanisms through which steroidal hormone signaling pathways mediate immune responses in insects.

Acknowledgments

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