Qiang Wang, Xiaoli Ju, Liang Chen and Keping Chen*

# Caspase-1 from the silkworm, *Bombyx mori,* is involved in *Bombyx mori* nucleopolyhedrovirus infection

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**Abstract:** Caspase-1 is one of the effector caspases in mammals that plays a central role in apoptosis. However, the lepidopteran caspase-1, especially the *Bombyx mori* caspase-1 (*Bm*-caspase-1), has not been investigated in detail. In this study, *Bm-caspase-1* was identified from an expressed sequence tag database in *B. mori* by BLAST search. The open reading frame of *Bm-caspase-1* contained 879 nucleotides and encoded 293 amino acids with a predicted molecular mass of 33 kDa. *Bm*-caspase-1 contained two consensus amino acid motifs of caspase cleavage sites, DEGDA and TETDG. Caspase activity assays revealed significant proteolytic activity of the Ac-DEVD-pNA substrate. *Bm-caspase-1* can be detected in all tissues and developmental stages by a semi quantitative polymerase chain reaction assay. More importantly, the expression level of *Bm-caspase-1* is increased upon baculovirus infection and up-regulated in BmNPV-resistant silkworms. Taken together, these results indicate that *Bm*-caspase-1 plays an important role during baculovirus infection.

**Keywords:** *Bm*-caspase-1; BmNPV; *BmNPV*-resistant silkworm; caspase activity.

## 1 Introduction

Apoptosis is a tightly regulated programmed cell death process leading to the elimination of unwanted cells. It plays a key role in embryonic development and tissue homeostasis [1]. Apoptosis is also a highly evolutionarily conserved process in different species. Diverse apoptotic stimuli, including viral infections, trigger apoptosis through several apoptotic pathways that converge on the activation of the caspase cascade. Caspases are a family of cysteine proteases that have long been considered to play important roles in programmed cell death [2]. They can be further classified into initiator and effector caspases based on their structure and order in the apoptosis pathways [3].

Since the identification of caspase-1 in 1992, several classes of initiator and effector caspases have been identified in different species ranging from vertebrate to invertebrate [2]. Three initiators and four effectors have been characterized in *Drosophila melanogaster* [4, 5]. Since the sequencing for the genome of the silkworm, *Bombyx mori* in 2004, it has become a key model organism for lepidopteran gene function and genetics studies [6]. Recently, five caspase family homologues have been identified in the *B. mori* genome, including two initiators (*Bm*Dredd and *Bm*Dronc) and three effectors (*Bm*-caspase-1, *Bm*-ICE, and *Bm*-caspase-N) [7]. However, the functions of *B. mori*'s caspases have not yet been clearly defined. *Bm*Dronc has been shown to be an initiator caspase responsible for the induction of caspase-dependent apoptosis [8]. *Bm*ICE-2 was identified as a novel pro-apoptotic gene with caspase-9 activity [9]. Caspase-1 is one of principal effector caspases and has been identified in multicellular organisms. It was recently reported that *Bm*-caspase-1 likely played a role in *B. mori* nucleopolyhedrovirus (BmNPV) infection in a study using comparative proteomic analysis [10]. However, the function of *Bm*-caspase-1 was not investigated in detail, especially in the context of baculovirus infection.

In this study, we cloned and characterized *Bm*-caspase-1 from the silkworm and found high similarity to the previously reported lepidopteran caspase-1. To examine its caspase activity, the full-length *Bm-caspase-1* gene was amplified and expressed in *Escherichia coli*. Cell lysates from *E. coli* expressing *Bm*-caspase-1 were examined using different synthetic substrates. The results showed that *Bm*-caspase-1 had significant proteolytic activity for the caspase-3 Ac-DEVD substrate. Furthermore, BmNPV infection increased *Bm-caspase-1* expression levels and activated caspase-1-like protease activity. Most importantly, real-time polymerase chain reaction (PCR) analysis indicated that *Bm-caspase-1* expression

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***Corresponding author: Keping Chen,** Institute of Life Sciences, Jiangsu University, Zhenjiang, Jiangsu, PR China, E-mail: kpchen@ujs.edu.cn
**Qiang Wang and Liang Chen:** Institute of Life Sciences, Jiangsu University, Zhenjiang, Jiangsu, PR China
**Xiaoli Ju:** School of Medicine, Jiangsu University, Zhenjiang, PR China

levels were much higher in BmNPV-resistant silkworms than in BmNPV-susceptible silkworms.

## 2 Materials and methods

### 2.1 Animals and real-time PCR analysis

Silkworms (NB, BC8, and 306 strains) were reared on fresh mulberry leaves at 25°C under 12:12-h light/dark cycles. Total RNA from *B. mori* larval tissues was extracted using the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA). The extracted RNA was used to synthesize cDNA using the PrimeScript™ RT reagent Kit (Takara, Kyoto, Japan) following the manufacturer's instructions. Semiquantitative PCR was performed according to the standard protocol using gene-specific primes and internal control Bm-rp49 primers. Gene expression was evaluated by real-time PCR as previously described [11]. Bm-rp49 primers were used as internal control.

### 2.2 Cloning of *Bm-caspase-1* and sequence analysis

Tn-caspase-1 sequence was used as target sequence to identify the *Bm-caspase-1* from *B. mori* expressed sequence tags (EST) database. Multiple sequence alignment was performed with BioEdit software (http://www.ebi.ac.uk/clustaw/). The phylogenetic tree was constructed in MEGA 5 software using neighbor-joining (NJ) method.

### 2.3 Bm-caspase-1 expression in *E. coli*

Gene-specific primers for *Bm-caspase-1* (forward: 5′-CGCGGATCCATGGCTGATGAAGAAAAGAAAACC-3′; reverse: 5′-AGTAGCGGCCGCCAAACAAGAGAAGGCGT-GTCAG-3′) containing *Bam*HI and *Xho*I restriction sites were synthesized (Shanghai Shenggong, Shanghai, China). The full length of *Bm-caspase-1* was amplified by PCR using pfu polymerase (Takara) and cloned into the pET-30a expression vector. After ligation, the recombinant vector was transformed into *E. coli* BL21. The expression plasmid was induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 28°C for 4 h.

Subsequently, the bacterial cells were centrifuged and resuspended in 50 mM phosphate buffer (pH 8.0). The bacterial cells were harvested by sonication and centrifugation and then they were boiled for 10 min. Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then stained with Coomassie brilliant blue.

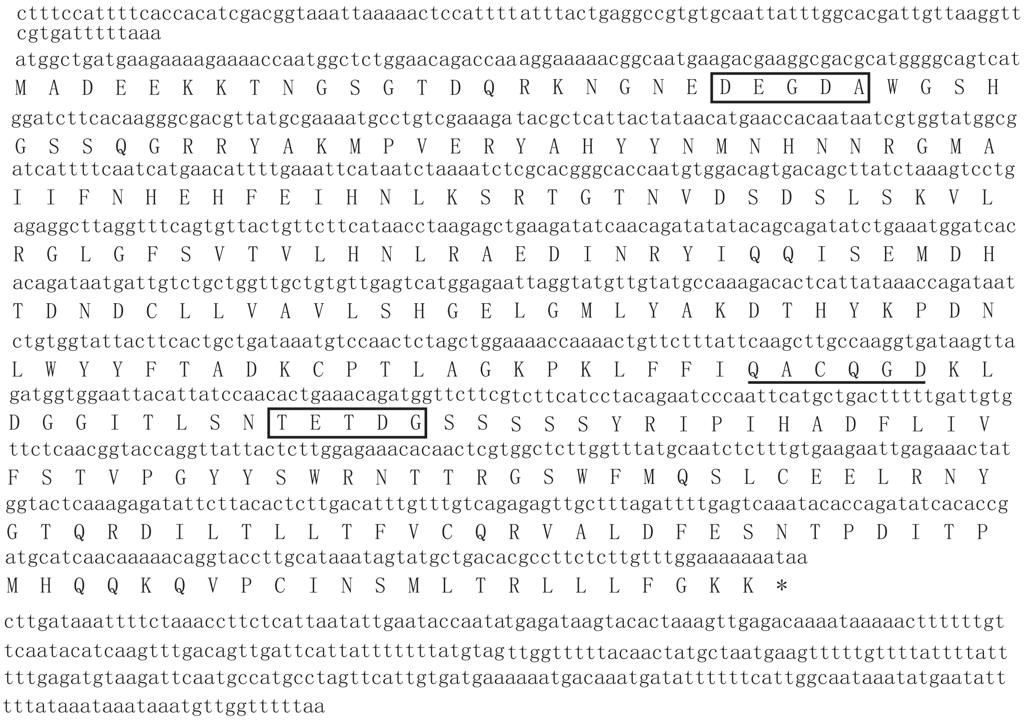
### 2.4 Caspase activity assay

Caspase activity was measured using a caspase activity kit according to the manufacturer's protocol (Beyotime, Haimen, China). After IPTG induction, cell lysates were prepared and the supernatants were incubated in a 96-well plate with Ac-DEVD-pNA, Ac-LEHD-pNA, or Ac-LETD-pNA at 37°C overnight. The absorbance values of pNA were measured at 405 nm using plate reader (BioTek, Santa Barbara, CA, USA).

## 3 Results

### 3.1 Isolation and characterization of Bm-caspase-1

The cDNA sequence showed high similarity to lepidopteran caspase-1 that was identified from the *B. mori* EST database using *Trichoplusia ni* caspase-1 sequence as a template. The open reading frame of this cDNA is 879 bp and encodes 293 amino acids with a putative molecular mass of 33 kDa (Figure 1). The Bm-caspase-1 protein sequence contained the highly conserved amino acid motif QACQG surrounding the catalytic site. It also contained two consensus motifs of caspase cleavage, DEGDA and TETDG (Figure 1). Protein sequence alignment results revealed that Bm-caspase-1 shared 83% identity with *Manduca sexta*, 81% identity with *T. ni*, 80% identity with *Chilo suppressalis*, and 79% identity with *Helicoverpa armigera* orthologues (Figure 2). As shown in Figure 2, the Bm-caspase-1 amino acid sequence shared a high level of identity among reported lepidopteran effector caspase-1 genes by multiple sequence alignment (Figure 2). All of these caspase-1 proteins displayed the highly conserved amino acid QACQG and two consensus caspase-cleavage motifs DEGDA and TETDG (Figure 2). Next, CLUSTALX and MEGA 5.0 phylogenetic analyses were performed to compare the Bm-caspase-1 with other species by the NJ method. As shown in Figure 3, Bm-caspase-1 was most closely related to Hm-caspase-1. The phylogenetic



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G  S  S  Q  G  R  R  Y  A  K  M  P  V  E  R  Y  A  H  Y  Y  N  M  N  H  N  N  R  G  M  A
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R  G  L  G  F  S  V  T  V  L  H  N  L  R  A  E  D  I  N  R  Y  I  Q  Q  I  S  E  M  D  H
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**Figure 1:** Sequence analysis of *Bm-caspase-1*: open reading frame sequence and amino acid sequences of *Bm*-caspase-1. Filled boxes, the two cleavage sites, DEGDA and TETDG, used to generate the large and small subunits; black lines, pentapeptide QACQG active site.

relationship analysis revealed that *Bm*-caspase-1 had closer orthologous relationships with other lepidopteran caspase-1 genes (Figure 3).

## 3.2 Expression and caspase activity analysis of Bm-caspase-1

We then expressed the complete ORF of *Bm-caspase-1* in *E. coli* BL21 using the vector pET-30a. After induction with 0.5 mM IPTG for 3 h, the recombinant protein was detected using SDS-PAGE analysis (Figure 4A). Cell lysates were prepared from *E. coli* BL21 and centrifuged at 16,200 *g* for 10 min at 4°C. The resultant supernatants were examined using different synthetic caspase substrates. The results showed significant proteolytic activity for the caspase-3 Ac-DEVD-pNA substrate. However, only negligible proteolytic activity was observed for the caspase-8 Ac-LETD-pNA and caspase-9 Ac-LEHD-pNA substrates (Figure 4B). Similar results were also observed using purified proteins as shown in Figure 4C. The results demonstrated that Bm-caspase-1 protein expressed in *E. coli* BL21 functions as an effector caspase.

## 3.3 Expression profiles of *Bm-caspase-1* in different tissues and developmental stages

Reverse transcription-PCR (RT-PCR) was used to determine the *Bm-caspase-1* mRNA expression levels in different tissues and developmental stages. *Bm-caspase-1* could be detected in all of the examined tissues including fat body, integument, head, midgut, silk gland, trachea, and hemocytes (Figure 5A). Next, the mRNA from day 1 of egg, pupae, silkworm moth, and first to fifth instars was isolated, and the *Bm-caspase-1* mRNA expression levels were examined. RNA from RT-PCR revealed that *Bm-caspase-1* was expressed in egg, pupae, silkworm moth, and first to fifth instars (Figure 5B). These results indicate that *Bm-caspase-1* is universally expressed in different tissues and at different developmental stages.

## 3.4 *Bm-caspase-1* mRNA expression levels increased during baculovirus infection

Previous studies have demonstrated that AcMNPV infection induced the expression and maturation of SI-caspase-1

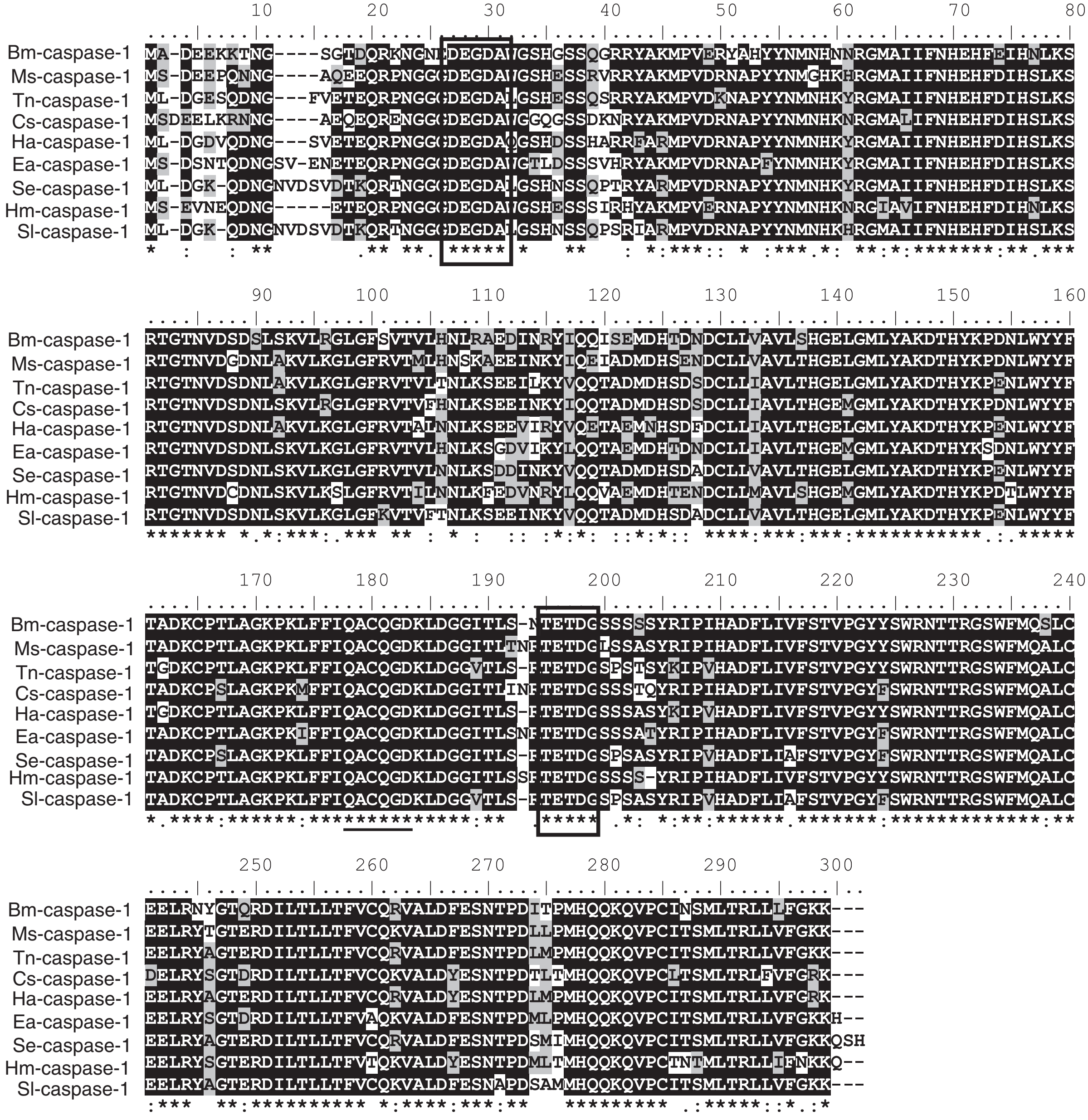

**Figure 2:** Amino sequence alignment of the *Bm*-caspase-1 with other lepidopteran caspase-1: multiple sequence alignment of caspase-1 from *Manduca sexta* (AEF30493), *Trichoplusia ni* (AAO17788), *Chilo suppressalis* (AFJ97219), *Helicoverpa armigera* (ABS18284), *Euphydryas aurinia* (AEF30498), *Spodoptera exigua* (AEK12771), *Heliconius melpomene* (ACU11588) and *Spodoptera litura* (BAM62940). Filled boxes, the two cleavage sites DEGDA and TETDG, used to generate the large and small subunits; black lines, pentapeptide QACQG active site.

in SL2 cells [12]. Silkworm larvae infected with BmNPV died with in 110 h post-infection [13]. However, no study has examined the caspase activity after infection by BmNPV in vivo. When fifth-instar *B. mori* larvae were infected with BmNPV, the mRNA expression levels of *Bm-caspase-1* in the midgut from different strains were significantly increased from 24 to 72 h compared with the control (Figure 6A). Next, the caspase activity was analyzed during the same 24 to 72 h post-infection. As shown in Figure 6B, the caspase activity of infected silkworms increased greatly after 72 h post-infection, but was not heightened at the time of infection. Taken together, these results demonstrated that *Bm-caspase-1* was likely involved in BmNPV infection of the silkworm.

## 3.5 *Bm-caspase-1* transcriptional level up-regulated in BmNPV-resistant silkworms

The differences in the 50% lethal dosage (LD50) values among silkworm populations were defined to be the

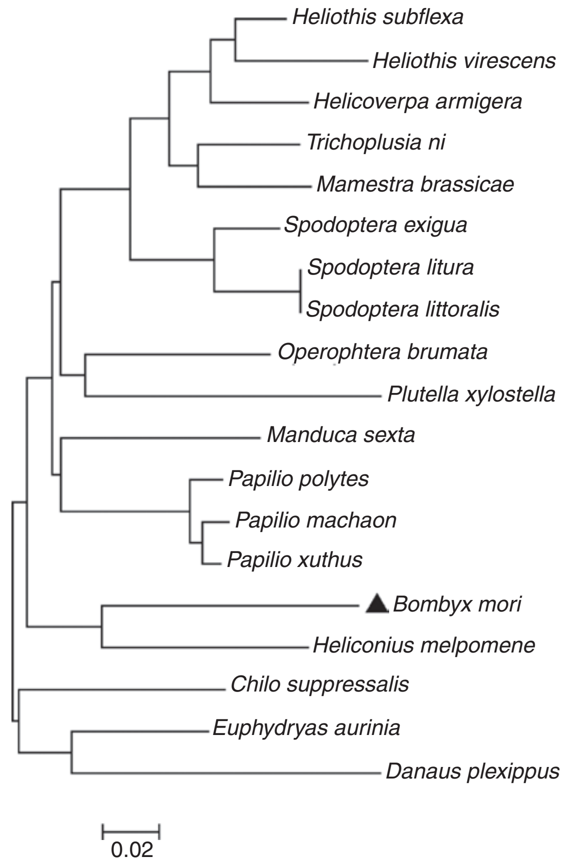

**Figure 3:** NJ phylogram showing the relationships between *Bm*-caspase-1 with respect to the other caspase-1. The phylogenetic tree was constructed in MEGA 5 software using the NJ method. ▲, Bm-caspase-1.

level of resistance to BmNPV infection. A silkworm strain named NB, with high resistance to BmNPV infection, was previously established. There was an approximately 1000-fold LD50 difference between NB and susceptible strain 306 after infection. A near-isogenic line named BC8 with resistance to BmNPV was also established [10, 14]. We then compared the mRNA expression levels of *Bm-caspase-1* between the two strains. As shown in Figure 7A, the Bm-caspase-1 transcriptional level in NB was much higher than in the susceptible strain 306, even at 0 h infection. At 48 and 72 h post-infection, *Bm-caspase-1* transcriptional levels were much higher in NB compared with the susceptible strain 306. Similar results were also observed in another resistant strain BC8 (Figure 7B). These results suggested that *Bm-caspase-1* probably played a critical role in silkworm resistance against BmNPV infection.

# 4 Discussion

In this study, after searching the silkworm genome database, we cloned and expressed *Bm*-caspase-1 into *E. coli* as a recombinant protein. The caspase enzyme activity assay revealed significant proteolytic activity for the caspase-3 Ac-DEVD-pNA substrate, but not the caspase-8 Ac-LETD-pNA or caspase-9 Ac-LEHD-pNA substrates.

We further found that the *Bm-caspase-1* expression level in the midgut was up-regulated during BmNPV infection of the silkworm. Furthermore, *Bm-caspase-1*

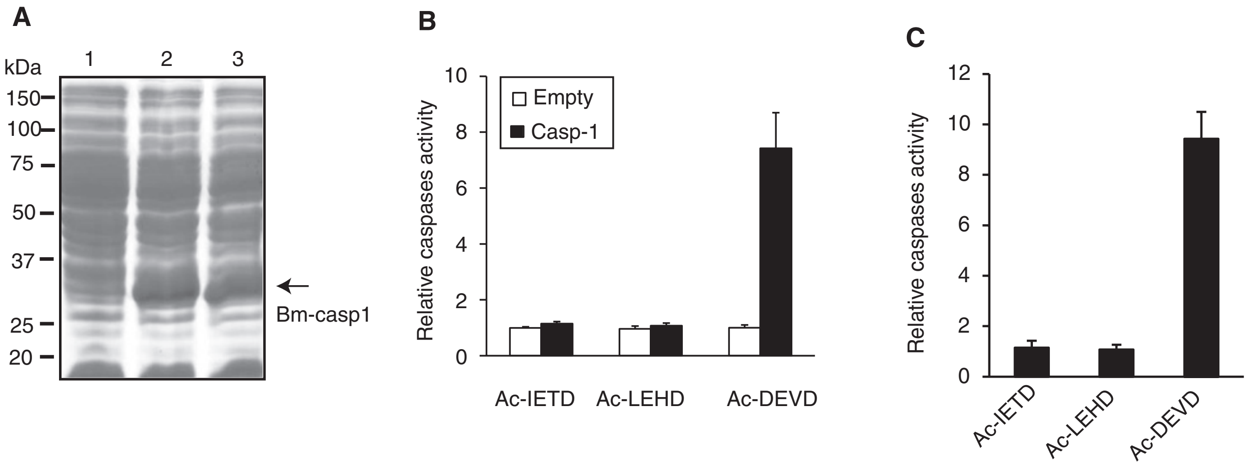

**Figure 4:** Caspase activity assay of Bm-caspase-1 in *Escherichia coli* BL21 cells. (A) Protein samples were separated by SDS-PAGE and stained with Coomassie brilliant blue. Lane 1, proteins from BL21 cells transformed with pET30a plasmid. Lanes 2 and 3, proteins from BL21 cells transformed with pET-30a-Bm-caspase-1. (B, C) Supernatants from BL21 cells or purified proteins were incubated with Ac-DEVD-pNA, Ac-IEHD-pNA, or Ac-LEHD-pNA at 37°C overnight. The absorbance values of pNA were measured at 405 nm. Relative caspase activity was calculated from the pNA standard curve.

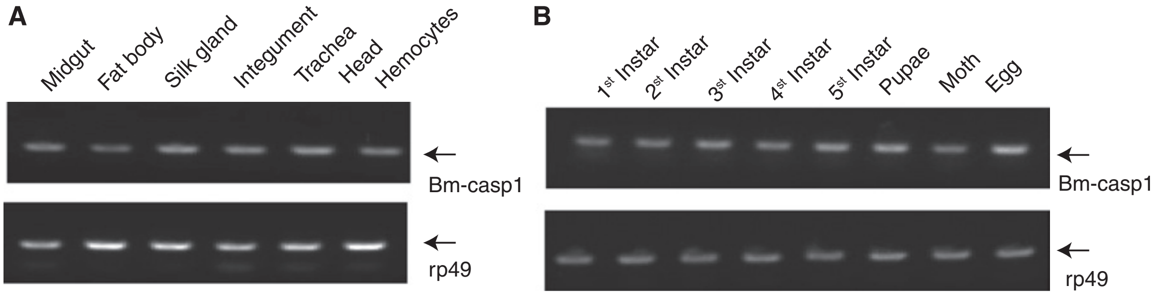

**Figure 5:** Transcriptional levels of *Bm-caspase-1* in different tissues and developmental stages. (A) *Bm-caspase-1* transcription levels in fat body, skin, head, midgut, silk gland, trachea, and hemocytes were measured by RT-PCR analysis. Rp49 was used as an internal control. (B) mRNA expression levels of *Bm-caspase-1* in the egg, pupae, silkworm moth, and first to fifth instars were measured by RT-PCR.

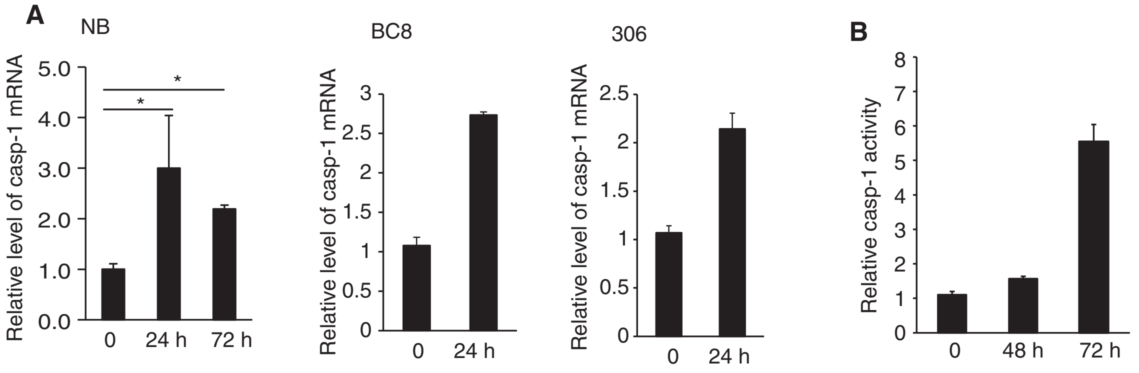

**Figure 6:** *Bm-caspase-1* transcription levels and caspase-1 activity in the midgut of silkworms after BmNPV infection. (A) BmNPV-resistant strain NB, BC8, and susceptible strain 306 were infected with BmNPV for 24 h or 72 h. Then, the midguts were isolated and mRNA was extracted for RT-PCR analysis. Rp49 was used as an internal control. (B). Protein samples from the midgut in the BmNPV-resistant strain NB were isolated and supernatants were incubated with Ac-DEVD-pNA at 37°C overnight. The absorbance values of pNA were measured at 405 nm. Error bars and asterisks indicate standard deviation and statistical significance, respectively. *$p<0.05$.

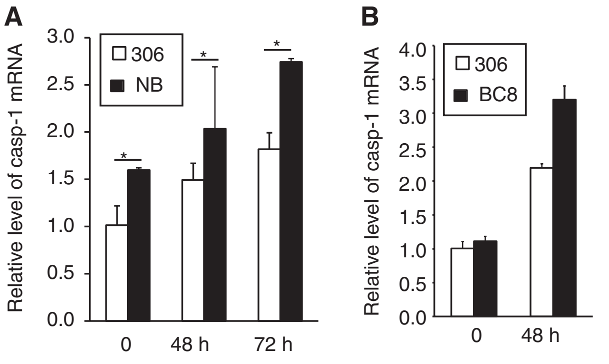

**Figure 7:** Relative expression levels of *Bm-caspase-1* in the BmNPV-resistant strain NB, BC8, and susceptible strain 306. (A, B) The BmNPV-resistant strain NB, BC8, and susceptible strain 306 were infected with BmNPV for 48 or 72 h. Fat bodies in the silkworm were isolated, and mRNA was extracted for RT-PCR analysis. Rp49 was used as an internal control. Error bars and asterisks indicate standard deviation and statistical significance, respectively. *$p<0.05$.

transcriptional levels in resistant strain NB were much higher than in the susceptible strain 306. Taken together, the data indicate that *Bm-caspase-1* plays an important role during baculovirus infection.

Apoptosis of host cells is known to play a critical role during baculovirus infection. At the early stage of infection, apoptosis serves as an antiviral defensive strategy to inhibit baculovirus replication and dissemination within the host. However, at the later stages of infection, apoptosis serves as part of the virus' strategy to self-propagate and promotes dissemination within the host [15]. A wild range of baculovirus infections induce apoptosis in different types of host cells, including *B. mori* BmN cells [16]. The apoptosis pathways in mammals have been extensively investigated; however, very little is known about apoptotic pathways in lepidopterans, especially during baculovirus infection. Inhibition of Sf-caspase-1 or Tn-caspase-1 expression significantly increased the production of recombinant proteins in the baculovirus expression vector system, suggesting that the apoptotic pathway mediated by caspase-1 plays critical role during baculovirus infection. Through an extensive survey of lepidopteran-derived EST datasets, Bm-caspase-1 was identified as an effector caspase; however, to date, the true function of Bm-caspase-1 remains obscure [7]. We found that Bm-caspase-1 contained two consensus motifs for caspase cleavage sites, DEGDA and TETDG. These two motifs were completely conserved among the lepidopteran caspase-1 proteins. Phylogenetic relationship analysis revealed that Bm-caspase-1 had closer orthologous relationships with lepidopteran caspase-1, suggesting that these caspase-1 proteins have similar functions. Both Ld-caspase-1 and Sf-caspase-1 were reported as effector caspases during baculovirus infection. Our results also confirmed that *Bm-caspase-1* was involved in BmNPV infection.

Many studies have investigated the genes involved in resistance against baculovirus infection. Reports show that the silkworm genes for serine proteases, BmNOX, and red fluorescent proteins had certain anti-BmNPV capabilities [17–19]. Although several genes, such as gloverin 3 and 4, arylphorin, and cathepsin B, were significantly up-regulated in the resistant silkworm strain after NPV infection, whether these genes have anti-BmNPV ability remains unknown. In this study, we also found that the caspase-1 transcriptional level in the resistant strain NB was much higher than in the susceptible strain 306. However, the full details of the molecular mechanisms involved in silkworm resistance against BmNPV infection need to be further investigated.

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