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Molecular cloning, expression and characterization of *Bmserpin-2* gene from *Bombyx mori*

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Serpins are a broadly distributed family of protease inhibitors. In this study, the gene encoding *Bombyx mori* serpin-2 (*Bmserpin-2*) was cloned and expressed in *E. coli*. The *Bmserpin-2* cDNA contains a 1125 bp open reading frame (ORF). The deduced protein has 374 amino-acid residues, contains a conserved SERPIN domain and shares extensive homology with other invertebrate serpins. RT-PCR analysis showed that *Bmserpin-2* was expressed in all developmental stages of *B. mori* larvae and various larval tissues. Subcellular localization analysis indicated that Bmserpin-2 protein was located in the cytoplasm. Interestingly, real-time quantitative PCR revealed that the expression of *Bmserpin-2* in the midgut of susceptible *B. mori* strain 306 significantly increased at 72 hours post inoculation (hpi) when infected with BmNPV. However, there was no significant increase of the *Bmserpin-2* expression in resistant strain NB infected with BmNPV. Thus, our data indicates that Bmserpin-2 may be involved in *B. mori* antiviral response.

Keywords: serpin-2, *Bombyx mori*, bioinformatics, subcellular location, qPCR

INTRODUCTION

Serpins are serine protease inhibitors that are widely distributed among animals, plants, viruses, and bacteria. They were initially described in 1980 (Hunt, 1980), later on they were demonstrated to play key roles in diverse biological processes *via* controlling the activity of proteinases. Recently, they have also been shown to be involved in insect immunity and other processes unrelated to proteinase regulation, inspiring another wave of exploration of new roles for serpins. The enzymatic mechanism for serpins to inhibit proteinase activity have been well characterized. One hallmark of this reaction is the behavior of the serpin reactive centre loop (RCL) (Irving *et al.,* 2000), a protein motif with a scissile bond between residues P1 and P1′ which can be cleaved by serine of the target protease and then the P1 residue will be affixed to the hydroxyl group in the active site (Huntington *et al.,* 2000; Dementiev, 2006). The P1 residue determines the primary selectivity of the serpin (Van Gent *et al.,* 2003). For example, many serpins with Lys or Arg at the P1 position inhibit trypsin (Gan *et al.,* 2001). Usually, the RCL is composed of approximately 20 amino acids near the C-terminus (Salzet *et al.,* 1999).

Insect serpins have been the focus of recent studies and shown to play roles in anti-pathogen immunological response. In *Manduca sexta*, the *serpin-1* gene encodes 12 isoforms generated by alternate splicing (Jiang & Kanost, 1997; Chamankhah *et al.,* 2003), and serpin-2 is found in hemocytes and its expression is up-regulated upon bacterial challenge, although their physiological roles remain elu-

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Abbreviations: BmNPV, *Bombyx mori* nucleopolyhedrovirus; hpi, hours post inoculation; IPTG, isopropyl β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; TIF-3, translation initiation factor 3 subunit 4.

sive (Gan *et al.*, 2001). *M. sexta* serpin-3, -4, -5 and -6 appear to prevent rampant oxidation of phenols and quinone formation by inhibiting the activity of prophenoloxidase activating proteases (PAPs) (Zhu *et al.*, 2003; Tong & Kanost, 2005). In *Anopheles gambiae*, *serpin-6* is expressed in the midgut and salivary glands during invasion by *Plasmodium berghei* (Abraham *et al.*, 2005). Human plasma also contains serpins that regulate serine protease cascades for processes such as blood coagulation and complement activation (Kanost, 1999; Silverman *et al.*, 2001; Pike *et al.*, 2005).

In this paper, we report the cloning and characterization of *Bombyx mori serpin-2* gene (*Bmserpin-2*, GenBank accession no.: AF242200). We carried out RT-PCR and subcellular localization analysis which showed Bmserpin-2 is a cytosolic protein expressed in all developmental stages of *B. mori* larvae and various larval tissues. Importantly, real-time quantitative PCR revealed that *Bmserpin-2* was expressed significantly in the midgut of susceptible *B. mori* strain 306 when infected with BmNPV, but not in resistant strain NB, indicating a possible role for Bmserpin-2 in the *B. mori* antiviral response.

MATERIALS AND METHODS

Insect, cell and virus. The standard silkworm strain C108, BmNPV-susceptible silkworm strain 306, BmNPV-resistant silkworm strain NB, BmN cell line, and BmNPV T3 isolate were maintained in our laboratory.

All larvae C108, 306 and NB were reared with fresh mulberry leaves at 25±2°C under a 12 h light/12 h dark photoperiod. Each newly molted 5th-instar larva of strain 306 and NB were inoculated *per os* with 1×10^6 BmNPV (5 μl, enough to get 100% infection in the 306 strain). These larvae were dissected, and the central and posterior regions of midguts were collected at 0, 6, 12, 24, 36, 48 and 72 hpi.

The BmN cell line was cultured at 27°C in TC-100 insect medium supplemented with 10% (v/v) fetal bovine serum (Gibco-BRL, Carlsbad) using standard techniques.

Bioinformatics analysis. Sequence analysis was carried out on-line at the website (http://www.ncbi.nlm.nih.gov and http://cn.expasy.org). In order to illustrate the genomic organization, the cDNA sequence was blasted to contigs of the *B. mori* genome in the GenBank, and SIM4 (http://pbil.univlyon1.fr/sim4.php) (Florea, 1998) was used to search for potential introns. The sequence comparison was conducted by BLAST program (National Center for Biotechnology Services, http://www.ncbi.nlm.nih.gov). A phylogenetic tree was constructed using MEGA version 4.1 (Tamura *et al.*, 2007) from CLUSTAL W alignments. The neighbor-joining method (Saitou & Nei, 1987) was used to construct the tree. The location of the gene on *B. mori* chromosomes was carried out online at the website (http://silkworm.swu.edu.cn/silksoft/silkmap.html).

RNA extraction and RT-PCR. Various tissues (testis, midgut, hemocytes, silk gland, Malpighian tubule, fat body, and ovary) from either the fifth instar C108 larvae, different developmental stages larvae of strain C108, or midguts of BmNPV-infected strains 306 and NB, which were dissected, then immediately frozen in liquid N_2 and stored at –80°C for later use. Total RNA was extracted from frozen samples with RNeasy mini kit (Qiagen), treated for 20 min at 37°C with RNase-free DNaseI (TaKaRa), purified with phenol/chloroform, precipitated with ethanol, and finally dissolved in DEPC-treated ddH_2O. cDNAs were generated from these RNA using M-MLV RTase (Promega) and an oligo-dT primer following the manufacturer's instructions.

To determine the tissue distribution of *Bmserpin-2* transcripts, the larval tissues of testis, midgut, hemocytes, silk gland, Malpighian tubule, fat body and ovary from strain C108 (fifth instar) were analyzed by RT-PCR. Similar experiments were also performed to compare the expression levels of *Bmserpin-2* gene in the different developmental stages of strain C108 larvae.

PCR was performed using the following primers for *Bmserpin-2*: Bmserpin-F: 5′ CGGGATCC (*Bam*HI) CCTGTTCTCAGTATGGATTCAAAG, Bmserpin-R: 5′ CCGCTCGAG (*Xho*I) ATTTCGTCCACGGTATTGGC 3′, where the underlined characters indicated restriction enzyme sites. A 284 bp fragment of *B. mori actin A3* (gene of *Bombyx mori* cytoplasmic actin A3) was amplified in parallel in each RNA sample using the following primers: Bm-actin A3-F: 5′ GCGCGGCTACTCGTTCACTACC 3′, Bm-actin A3-R: 5′ GGATGTCCACGTCGCACTTCA 3′ as an internal control. The PCR reaction was carried out for 35 amplification cycles (94°C/60 s, 58°C/45 s, and 72°C/90 s) in a Gene Amp 2400 System thermocycler. Agarose gels (1%) were used for electrophoreses.

Protein expression and purification. The above PCR product was ligated into pMD18-T vector (TaKaRa) using T4 DNA ligase (TaKaRa), and then transformed into *E. coli* (TG1 strain). The plasmid pMD18-T/serpin-2 was digested with *Bam*HI and *Xho*I, and then the purified fragment was subcloned into the pET30a (+) expression vector (Novagen, USA) in frame with the N-terminal 6×His tag. pET30a (+)/serpin-2 was transformed into *Escherichia coli* (BL21 strain) for protein expression.

For expression of recombinant protein, a positive clone was cultured in Luria-Bertani (LB) medium supplemented with kanamycin (50 μg/ml) over-

night at 37°C with shaking. This culture was added into fresh LB medium and cultured at 37°C with vigorous shaking to OD_{600} of 0.6. The culture was then induced with IPTG (final concentration of 0.2 mM) and further cultured for another 5 h at 37°C. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE, 12%) was used to analyze the recombinant protein. SDS/PAGE was performed in the Mini-Protean system (Bio-Rad, USA). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R250 to visualize the protein bands.

According to the protocol for mass spectrography as described by instruction, briefly, the specific bands were excised manually from the gel with a sterile scalpel and digested with trypsin. The digested samples were analyzed using an ultraflex II MALDI TOF MS (Bruker, Germany). Peptide mass fingerprinting was performed using MASCOT search engine (http://www.matrixscience.com) against the NCBI protein database to definite the protein.

Antibody production. The 6×His-tagged recombinant Bmserpin-2 protein was purified on a Ni^{2+}-NTA column (Novagen) and used to raise polyclonal antibodies in rabbits. The antibody was prepared using standard techniques. Purified Bmserpin-2 protein (about 2 mg) was injected subcutaneously to immunize New Zealand white rabbits in complete Freund's adjuvant, followed by two booster injections in incomplete Freund's adjuvant within a gap of 2 weeks before exsanguinations. The polyclonal rabbit antibody against 6×His-Bmserpin-2 was used for immunoassay.

Immunofluorescence microscopy. BmN cells seeded onto coverslips were washed with PBS, and fixed with 2 ml of 4% paraformaldehyde for 15 min. Then cells were washed three times with PBS and permeabilized with 0.1% Triton X-100 in PBS for 15 min. After washing three times with PBS, cells were incubated with anti-Bmserpin-2 antibody (1:1000) as primary antibody, fluorescein isothiocyanate (FITC) -conjugated goat anti-rabbit IgG antibody as secondary antibody (1:3000) (Qualex, Inc), and examined with a Leica confocal laser scanning microscope.

Real-time quantitative PCR. To compare the *Bmserpin-2* gene expression levels of BmNPV-infected strains 306 and NB, real-time quantitative PCR was carried out using the Mx3000PTM PCR instrument (Stratagene). A 147 bp product for *Bmserpin-2* cDNA was amplified using the following primers: forward 5′ AAATACCTCGACAGCGTCCTTCT 3′ and reverse 5′ AAATACCTCGACAGCGTCCTTCT 3′. A 192 bp product of silkworm housekeeping gene *TIF-3* was amplified as an internal control with the same templates by using the following primer set: forward 5′ AGATGACGGGGAGCTTGATGGT 3′ and reverse 5′ GAGGGCGGAATGTACTTGTTGC 3′. The thermal cycling profile consisted of initial denaturation at 95°C for 3 min and 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The PCR mixture (10 μL) contained 1 × SYBR GREEN PCR mix (TaKaRa), 0.2 μL ROX reference Dye (50 ×), 1 μL of each primer (1 μM), and 2 μL of 1:10 diluted cDNA templates, according to the manufacturer's instructions. The specificity of PCR amplification was determined by constructing a melting curve after the PCR amplification. Melting curve analysis was performed in the range of 55–95°C by monitoring SYBR Green fluorescence with 0.5°C increment. Each sample was processed in triplicate. The relative expression ratios of the gene were calculated relative to the housekeeping gene using the comparative *Ct* (2-°*Ct*) method (Wong & Medrano, 2005). The data was analyzed using SPSS 13.0.

RESULTS

Sequence analysis of *Bmserpin-2*

The sequence of a 1125 bp *B. mori* cDNA encoding Bmserpin-2 was constructed *in silico* through NCBI, which encods a protein of 374 amino-acid residues with calculated molecular mass of 41.8 kDa and predicted isoelectric point of 4.87. This gene locates in the 15th silkworm chromosome, and contains eight exons and seven introns. The first exon (E1) was found in contig 2640, others are in contig 2639 (Fig. 1).

According to the phylogenetic tree analysis (Fig. 2), the evolution of *Bmserpin-2* was in accordance with other species. *Bmserpin-2* has high homology with *serpin-2* from other insect species. It was submitted to NCBI for PSI-BLAST searching and the result showed that *Bmserpin-2* gene has 42% identity with *B. mori* antitrypsin, 55% identity with *Manduca sexta serpin-2*, and is highly similar to *serpin-2* from *Lonomia obliqua* (lepidoptera, Identities = 70%, Positives = 86%), *Spodoptera exigua* (lepidoptera, Identities = 66%, Positives = 82%), *Ctenocephalides felis* (Siphonaptera, Identities = 40%, Positives = 61%), also 33% identity with *Branchiostoma lanceolatum* (cephalochordata) *serpin-2* precursor.

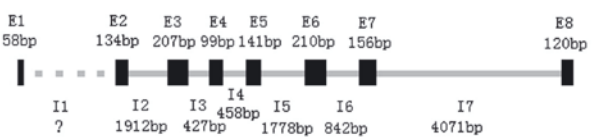


Figure 1. Genomic organization of *Bmserpin-2*.
It is based on contig 2640 (GenBank accession no.: BABH01002640) and contig 2639 (GenBank accession no.: BABH01002639). The length of intron 1 (I1) is unclear, exon 1 (E1) was found in contig 2640, exons 2–8 (E2–E8) were found in contig 2639.

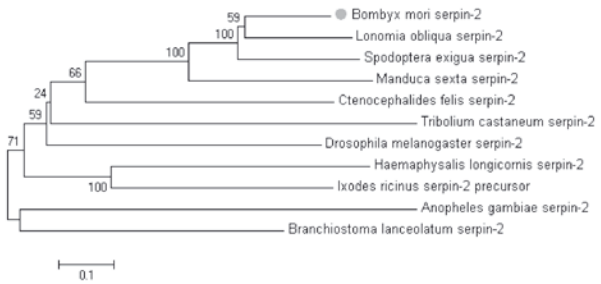


Figure 2. Phylogenetic analysis of serpin-2 from insect and other species.
Basing on MEGA version 4.1 analysis of CLUSTAL W alignments. The neighbor-joining method was used to construct the tree. The serpin-2 used in analysis included *S. exigua* (GenBank accession no.: ABU62829), *C. felis* (GenBank accession no.: AAN73324), *L. obliqua* (GenBank accession no.: AAV91429), *M. sexta* (GenBank accession no.: AAB58491), *H. longicornis* (GenBank accession no.: BAD11156), *A. gambiae* (GenBank accession no.:ABJ52801), *B. mori* (GenBank accession no.: AF242200), *D. melanogaster* (GenBank accession no.: CAB63097), *I. ricinus* (GenBank accession no.: ABI94056), *B. lanceolatum* (GenBank accession no.: CAJ55430), *T. castaneum* (GenBank accession no.: XP_974313).

Bmserpin-2 contains only one conserved signature motif SERPIN, the amino-terminal end of which lacks a hydrophobic secretion signal, indicating that it may be an intracellular protein. As predicted, *L. obliqua* and *C. felis* serpin-2 have a signal peptide. Bmserpin-2 contains an Arg residue at its predicted P1 position (Fig. 3), which suggested that it may inhibit enzymes with trypsin-like specificity (Gan *et al.*, 2001).

Developmental and tissue expression pattern of *Bmserpin-2*

The levels of *Bmserpin-2* in various developmental stages of silkworm were examined by RT-PCR (Fig. 4A). The results indicated that its mRNA was detectable from egg to fifth instar larvae. To investigate its expression profile, the total RNA from fifth instar larvae was isolated from testis, ovary, hemocytes, fat body, midgut, silk gland and Malpighian tubules, and subjected to RT-PCR. The results showed *Bmserpin-2* gene expression could be detected in all tissues (Fig. 4B).

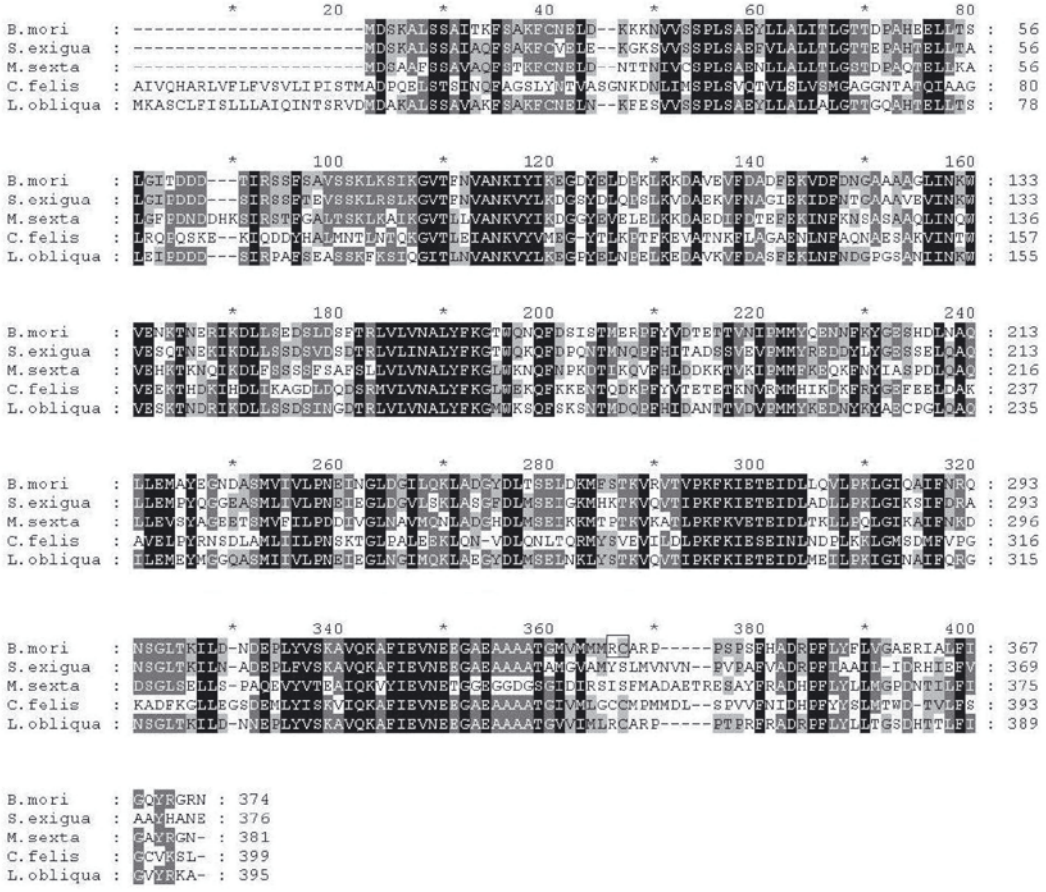


Figure 3. Multi-sequence alignment of serpin-2 form *B. mori* and other insects.
Identical amino acids are indicated in white with black background and conserved amino acids in white with gray background. The predicted P1–P1′ position is marked by "□" at position 366–367. The aligned serpin-2 were from *S. exigua* (GenBank accession no.: ABU62829), *M. sexta* (GenBank accession no.: AAB58491), *L. obliqua* (GenBank accession no.: AAV91429), *C. felis* (GenBank accession no.: AAN73324).

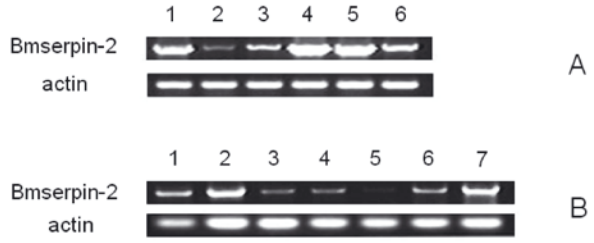


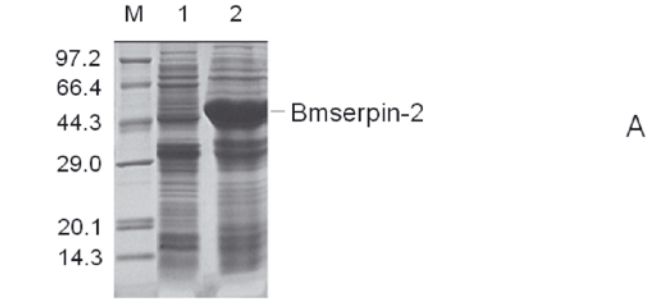
Figure 4. *Bmserpin-2* expression in development and different tissues of silkworm.
A. RT-PCR analysis of *Bmserpin-2* in different development stages of *B. mori*. Lanes 1–6 represent egg, first instar, second instar, third instar, fourth instar and fifth instar. **B**. Expression profile of *serpin-2* in different tissues of *B. mori*. Lanes 1–7 represent testis, midgut, hemocytes, silk gland, Malpighian tubule, fat body, and ovary respectively. *Bmactin A3* was used as the control to normalize the amount of templates.

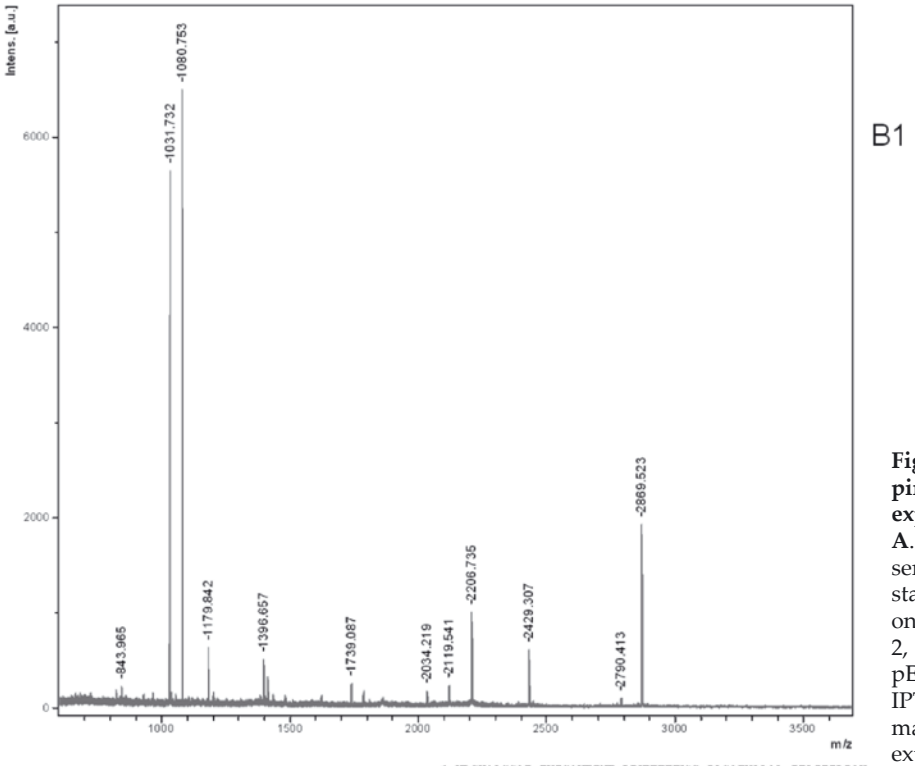
Protein expression and characterization by SDS/PAGE and mass spectrography

Bmserpin-2 was successfully expressed in *E. coli* (BL21 strain) as a fusion protein. Since the fusion protein had a His-tag at the N-terminus and C-terminus, its molecular mass was 47.3 kDa which was higher than that of the predicted mass (Fig. 5). Unfortunately, recombinant Bmserpin-2 expressed in *E. coli* was predominantly insoluble (not shown). This protein was further characterized by by MALDI-TOF analysis with 36% amino acid coverage (Fig. 6).

Subcellular localization of Bmserpin-2

The intracellular localization of Bmserpin-2 was determined by immunofluorescence using pre-





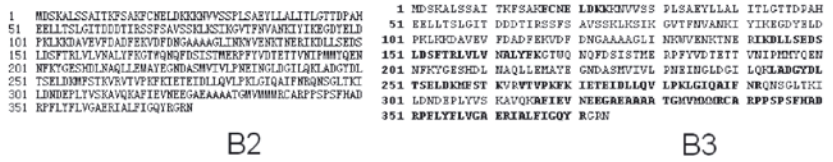


Figure 5. Expression of Bmserpin-2 in *E. coli* and identify the expressed protein.
A. Expression of recombinant Bmserpin-2 in *E. coli*. Lane 1, BL21 star transformed with pET30a only and induced by IPTG; Lane 2, BL21 star transformed with pET30a-serpin-2 and induced by IPTG; M, Protein molecular mass marker. **B**. Identification of the expressed protein by MALDI-TOF analysis. **B1**. Peptide sequences identified by mass spectrometry. **B2**. Amino-acid sequences of Bmserpin-2. **B3**. Matched peptide sequences are shown as bold characters.

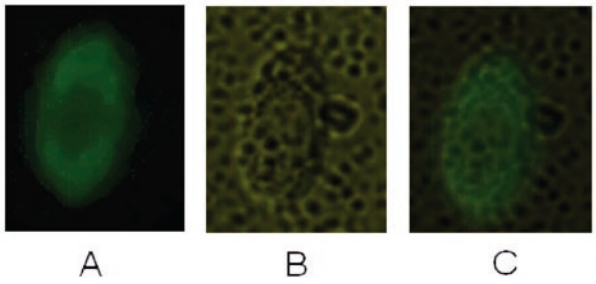


Figure 6. Subcellular localization of Bmserpin-2 in BmN cells.
BmN cells were treated with anti-Bmserpin antibody, followed by treatment with FITC-conjugated goat anti-rabbit IgG. Left to right: green fluorescence for Bmserpin-2, bright field, and merged images.

pared antiserum. We could see that Bmserpin-2 may localize primarily in the cytoplasm (Fig. 6).

Real-time quantitative PCR

Through real-time PCR, we found that following infection with BmNPV of strain 306 the quantity of *BmSerpin-2* mRNA was significantly increased at 72 hpi, and at this time larva of BmNPV-infected strain 306 showed some symptoms characteristic of disease (not shown). However, it was not significantly increased in larva of BmNPV-infected strain NB from 0 to 72 hpi (Fig. 7). Melting curve analyses showed the presence of a single PCR product for either *Bmserpin-2* or *B. mori TIF-3*, confirming the specificity of the reaction (not shown).

DISCUSSION

Proteases and protease inhibitors play essential roles in numerous organisms and various cellular processes. Serpins are a widely distributed family of serine protease inhibitors that have been shown

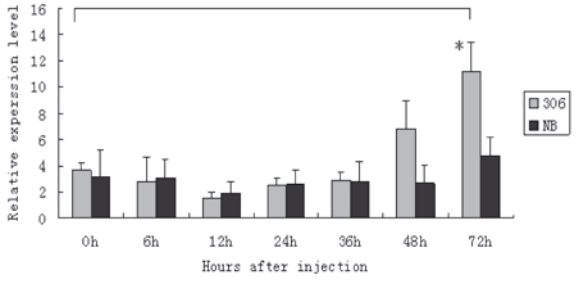


Figure 7. Real-time PCR analysis of Bmserpin-2 in midgut of susceptible strain 306 and resistant NB in response to BmNPV challenge.
Fifth instar larvae were injected with BmNPV and samples were taken at different times post inoculation. *Student's *t*-test, $P<0.05$.

to be implicated in host-pathogen interaction, such as insect anti-virus immunity (Gan *et al.*, 2001). As far as we know, currently there are no other publications about the characterization and functional analysis of *B. mori* serpins. Thus, our work presented here represents the first report about cloning and characterization of a *B. mori* serpin, designated as *Bmserpin-2*. We also provided evidence that Bmserpin-2 may be involved in *B. mori* anti-BmNPV response.

Sequence analysis revealed significant homology of *Bmserpin-2* with *serpin-2* from other insect species, indicating a highly conserved and similar role for these serpins. RT-PCR analysis showed *Bmserpin-2* was expressed in all development stages and in all tissues from *B. mori* larvae, suggesting the essentiality of their activity. We further successfully expressed Bmserpin-2 in *E. coli* and characterized the protein by SDS/PAGE and mass spectrography. Finally, our subcellular localization analysis showed that Bmserpin-2 was a cytoplasmic protein.

An important obstacle for sericulture is the BmNPV infection, which can cause devastating damage to *B. mori* in tropical regions, as happended in recent years (Khurad *et al.*, 2004). It has become increasingly important to delineate the silkworm-BmNPV interaction to facilitate development of anti-BmNPV therapy. Many proteases purified from the silkworm have been shown to have anti-virus activity, for example, a serine protease purified from larval digestive juice has anti-BmNPV activity *in vitro* (Nakazawa *et al.*, 2004). As serpin-2 from *M. sexta* can inhibit human neutrophil cathepsin G (a serine proteinase) (Gan *et al.*, 2001), Bmserpin-2 may also be able to inhibit serine proteases because they have high homology. So the significant increase of *Bmserpin-2* expression in BmNPV-infected strain 306 may be one strategy employed by the virus to help block host protease activity. Interestingly and reasonably, the expression of *Bmserpin-2* did not change much in BmNPV-treated strain NB, indicating that this pathway hijaked by BmNPV was disrupted *via* some unknown mechanism. Taken together, our data provides evidence that Bmserpin-2 may play a role in host anti-virus response, and our characterization and protein expression analysis pave the way for subsequent studies.

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