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Characterization and functional analysis of serpin-1 like gene from oak silkworm *Antheraea pernyi*

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Abstract

Serpins are a broadly distributed family of proteases found in various organisms that play an important role in regulating the immune response. Here, we identified a serpin-1 gene from *Antheraea pernyi* that encodes a 279 amino acid protein with a molecular weight of 30.8 kDa. We expressed the recombinant Ap-serpin-1 protein in *Escherichia coli* and used the purified protein to prepare rabbit anti-Ap-serpin-1 polyclonal antibodies. We calculated the enzyme-linked immunosorbent assay titer of the antibody as 1:128000. Quantitative real-time polymerase chain reaction analysis revealed that Ap-serpin-1 was expressed in all examined tissues, including hemolymph, malpighian tubules, midgut, silk gland, integument and the fat body; the highest Ap-serpin-1 expression levels was detected in the fat body. We next investigated the expression patterns of Ap-serpin-1 in both fat body and hemolymph samples, following treatment with *E. coli, Beauveria bassiana, Micrococcus luteus* and nuclear polyhedrosis virus (NPV). We reported that NPV and *M. luteus* significantly enhanced Ap-serpin-1 expression in the fat body. While, in the hemolymph samples, treatment with *B. bassiana* and *M. luteus* was shown to upregulate Ap-serpin-1 expression at 24 h induction. Altogether, our results suggest that Ap-serpin-1 is involved in the innate immunity of *A. pernyi*.

Keywords: *Antheraea pernyi*, serpin-1, innate immunity, expression

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Introduction

Serine protease inhibitors belong to the serpin superfamily, acting mainly as structurally conserved suicidal inhibitors (Yamasaki *et al.*, 2010). Over 70 serpin structures have been determined; these have been characterized as 40–50 kDa plasma proteins with domain typically consisting of 3 β-sheets and 8–9 α-helices. Serpins undergo a drastic conformational change and form a stable complex with the protease (Abraham *et al.*, 2005) to inhibit protease function (Law *et al.*, 2006). Although most serpins function to inhibit serine proteases, some inhibit other types of protein enzymes like cysteine protein enzymes (Gettins, 2002). Moreover, serpins that are more prevalent in invertebrates appear to be generated through alternative exon splicing (Zou *et al.*, 2009).

Serine protease inhibitors have now been identified in many kingdoms of life ranging from microorganisms to larger animals and have been shown to play a critical role in blood coagulation, complement activation, melanotic encapsulation and Spätzle processing (Irving *et al.*, 2000). In insects, resembling serpins were first isolated from the hemolymph of silkworm larvae (Sasaki & Kobayashi, 1984) and were shown to participate in the immune response mediated by prophenoloxidase (proPO) activation. Mediated by serine protease, proPO is triggered by a cascade of proteolytic cleavage sites to generate phenoloxidase (PO), which oxidizes phenolic compounds to produce melanin (Bilang Liu *et al.*, 2012). Therefore, when microorganisms invade, proPO localizes melanization to the surface of invading microorganisms (Tong *et al.*, 2005).

In *Manduca sexta*, serpins inhibit hemolymph proteases (HPs) involved in the defense response against pathogen infection and tissue damage (Kanost, 1999; Jiang, 2008). *Arabidopsis*

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thaliana serpins inhibit metacaspase-9 cysteine protein enzyme (Irving *et al*., 2000). In *Bombyx mori*, serpin-15 (Bmserpin-15) was shown to inhibit prophenoloxidase activation in *B. mori* hemolymph, while injection of Bmserpin-15 significantly reduced transcript levels of antimicrobial peptides in the fat body (Liu *et al*., 2015). In *Ostrinia furnacalis*, the levels of serpin-1 transcripts in the integument were higher and shown to be inhibited by challenges with *Staphylococcus aureus* and *Escherichia coli* (Zhang *et al*., 2016). Furthermore, Spn77B was shown to induce systemic expression of the antifungal peptide Drosomycin via the Toll pathway (Tang *et al*., 2008). These results demonstrate that serpins are an important factor involved in insect innate immunity.

Antheraea pernyi is an important silk-producing insect that lives in the wild. Although the organism exhibits significant advantages in disease resistance, disease outbreaks continue to cause severe economic loss each year. Hence, there is an urgent need to improve disease resistance in *A. pernyi* against pathogens. Fat bodies and hemolymph are considered important immune organs in insects and are involved in the immune response (Sun *et al*., 2015). Therefore, in this study, we conducted characterization, tissue distribution and expression patterns of serpin-1 in *A. pernyi* following various microorganism challenges. Our results indicate that Ap-serpin-1 might be involved in the innate immune responses of *A. pernyi*.

Materials and methods

Experimental animals

The Sericultural Research Institute of Henan, China, provided *A. pernyi* specimens for our experiments. We fed fresh leaves to the 1st through 4th instar larvae, indoors, three times a day, at 25°C, with humidity ranging from 65 to 75% and a photoperiod of about 14 h:10 h (light: dark). For our experiments, we sampled tissues from the midgut, silk gland, fat body, malpighian tubules, integument and hemolymph (Zhang *et al*., 2015).

Total RNA extraction and cDNA synthesis

We isolated total RNA from the fat body of *A. pernyi* 5th instar larvae using Trizol Reagent (Takara, Dalian, China), and followed the manufacturer's protocol. We synthesized the first-strand cDNA using the PrimeScript™ One Step real-time polymerase chain reaction (RT-PCR) Kit Ver.2 (Takara, Dalian, China), following the manufacturer's instructions. We checked the quality of cDNA by electrophoresis on a 1% agarose gel and spectrophotometry and stored the cDNA at −80°C until usage.

Cloning and sequence analysis of Ap-serpin-1

We obtained *A. Pernyi serpin-1* cDNA fragment (Genbank succession number KY322717) from the cDNA library, which we constructed in our laboratory. We designed primers serpin-1F1 and serpin-1R1 (table 1) to amplify the *Ap-serpin-1* cDNA and performed PCR using the following conditions: 10 min initial denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min 20 s, before a final elongation step at 72°C for 10 min. We analyzed the PCR product by 1% agarose gel electrophoresis, before verifying the sequence at Huada Company. We used DNAstar software (version 5.02) to predict the open reading frame (ORF). We determined the signal peptide using SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP-4.1/). We calculated the molecular weight of the Ap-serpin-1 protein using ExPASy (http://web.expasy.org/compute_pi/) and performed multiple sequence alignments using ClustalX2 (http://www.ebi.ac.uk/tools/clustalw2/index.html). We then constructed a phylogenetic tree by MEGA 5.1 using the neighbor-joining algorithm method (Tamura *et al*., 2011) and performed statistical analysis using 1000 bootstrap replicates.

Expression and purification of recombinant of Ap-serpin-1 protein

We designed primers Ap-serpin-1-F1 and Ap-serpin-1-R1 (table 1) to amplify the *Ap-serpin-1* cDNA sequence at the ORF and performed PCR as follows: 5 min initial denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min 20 s, and final elongation step at 72°C for 10 min. We purified the PCR product using a DNA Gel Extraction Kit (Axygen, Hangzhou, China) before ligating with PMD-19 T vector to obtain plasmid *PMD-19T-Ap-serpine-1*. After digestion with EcoR I and Sal I (Takara), we ligated the *Ap-serpine-1* fragment into *pET-28a* (+) vector for protein expression in *E. coli* (Transetta DE3) cells (TransGen Biotech, Beijing, China). We used isopropyl-β-D-thiogalactopyranoside (IPTG) and induced protein expression for 5 h at 37°C. We harvested *E. coli* cells by centrifugation at 8000 × *g* for 10 min at 4°C, which we then completely lysed by sonication. Following further centrifugation at

Table 1. Sequences of primers used in this study.

Primer name	Primer sequence (5′−3′)	Purpose
serpin-1F1	GTCAACATGAAGATTACAGTTTGCA	amplification open reading frame
serpin-1R1	AATGGACGGTTCTGGGTTA	
Ap-serpin-1-F1	**CCGGAATTC**ACAGACTTACAAACA	Recombinant expression
Ap-serpin-1-R1	**GCGTCGAC**CTAGAGATTCCGCT	
QAp18s F	ATTTTTGCGGTCTTGTTCGT	Real-time polymerase chain reacation
QAp 18s R	TCCGCAATGTGTCTTTGTGT	
QApSerpin-1 F2	GCCAAAGCAAACCCAGATAA	
QApSerpin-1 R2	GAATTTTGCAAATGGCGAGT	

Note: Restriction sites are underlined.

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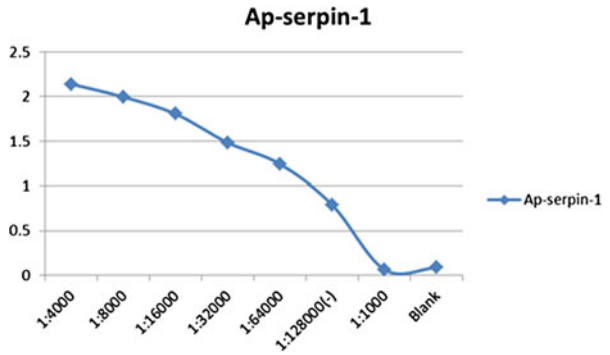

Fig. 1. ELISA titer determination.

12,000 × *g* for 15 min at 4°C, we purified the recombinant protein using a Ni-NTA Agarose column (QIAGEN, China) under native conditions, according to the manufacturer's protocol. We analyzed purified recombinant protein using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting using an anti-FLAG antibody. We determined the protein concentration using the Bradford assay (Bradford, 1976).

Antibody preparation and titer determination

We prepared anti-Ap-serpin-1 polyclonal antibodies according to the method (Harlow & Lane, 1999) previously described. Briefly, we incubated 600 μg of purified protein with Freund's complete adjuvant and immunized male New Zealand rabbits. After 2 weeks, we used the purified protein diluted with Freund's incomplete adjuvant for the second immunization. We collected the antiserum after the third immunization boost and used western blotting to confirm the specificity of the antibodies. We determined the titer of the antibody using enzyme-linked immunosorbent assay (ELISA) (Buss *et al.*, 1997). Briefly, we incubated Ap-serpin-1 with coated solution at 4°C overnight. After washing with PBST (blocking with 5% non-fat milk diluted with PBS containing 0.1% Tween-20), we incubated the recombinant protein with the primary antibody (diluted 1:1000, 1:4000, 1:8000, 1:16000, 1:32000, 1:64000, 1:128000, respectively) at 37°C for 2 h. Then, we incubated the recombinant protein with goat anti-rabbit IgG (diluted 1:2000 with PBST; Beyotime, Shanghai, China) for 2 h at 37°C. Finally, we added the TMB substrate solution and used an ultraviolet spectrophotometer to measure optical density (OD) at 450 nm. The results of ELISA showed that the titer of Ap-serpin-1 antibody was 1:128000 (fig. 1).

Expression of Ap-serpin-1 in various tissues and developmental stages

To determine the tissue-specific expression of Ap-serpin-1, we collected midgut, silk gland, fat body, malpighian tubules, integument and hemolymph from the third day of the 5th instar larvae. Moreover, we used the fat body (from 1st to 5th instar) from the larvae, pupae, moth and spawn to investigate the relative expression level of Ap-serpin-1 at different developmental stages. We extracted total RNA using the TRIzol reagent (Takara) and the first strand cDNA was synthesized using PrimeScript™ RT Master Mix (Takara). The primers used for PCR were designed using the online tool Primer3 online tool (http://bioinfo.ut.ee/primer3-0.4.0/) (table 1). We performed RT- PCR as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 57°C for 30 s, and a final extension at 72°C for 30 s. We applied a melting curve analysis (65–95°C) to confirm the unique and specific PCR products for each reaction. We determined the relative expression levels according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). We normalized all quantitative RT-PCR (qRT-PCR) experiments using *A.*

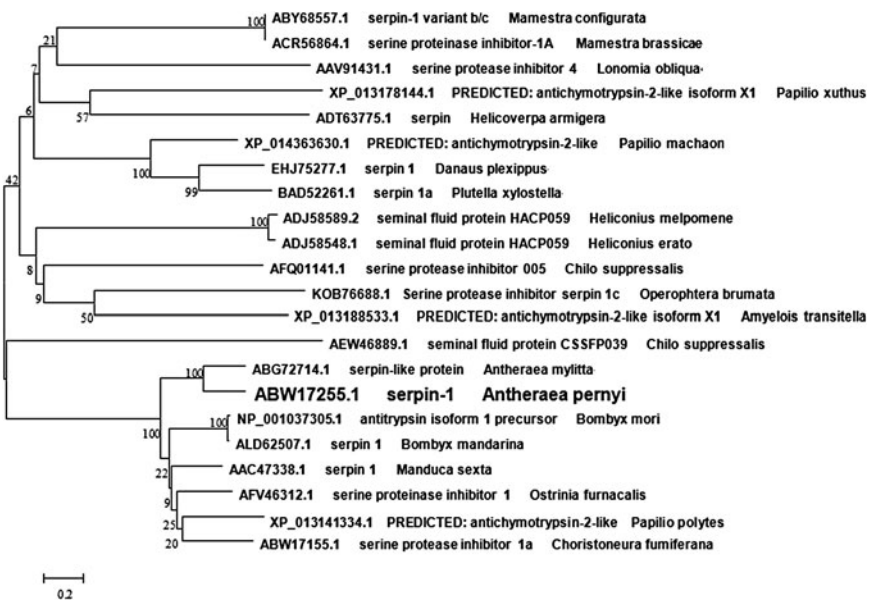

Fig. 2. Phylogenetic analysis of *Ap-serpin-1* with serpins from other species. The phylogenetic tree was constructed using the neighbor-joining algorithm and bootstrap values (1000 repetitions) of the branches are indicated. The name and GenBank accession numbers of the serpins are indicated.

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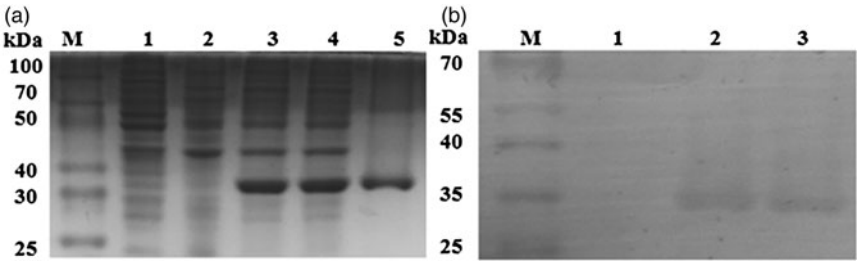

Fig. 3. Expression and purification of recombinant Ap-serpin-*1*. (a) Analysis of recombinant *A. pernyi* serpin-1 proteins on 12% SDS-PAGE gels. Bacterial proteins were collected after 4 h of induction with different isopropyl-β-D-thiogalactopyranoside (IPTG) concentrations. M: protein molecular weight marker; Lane 1, *PET-28a* (+) control without recombinant proteins in *E. coil* (Transetta DE3) cells; Lane 2, non-induced cells; Lane 3, induction by 0.5 mM IPTG; Lane 4, induction by 1.0 mM IPTG; Lane 5, purification of recombinant protein. (b) Western blotting analysis of recombinant proteins using anti-His-tag antibodies. M: protein marker: Lane 1: non-induced cells: Lane 2: induction by 0.5 mM IPTG: Lane 3: induction by 1.0 mM IPTG.

pernyi 18s rRNA gene as an internal control. We repeated each biological treatment three times and presented the data as the means ± standard error.

Western blotting

We selected tissues for total protein extraction using RIPA lysis buffer (Aidlab Biotech, Beijing, China) and determined protein concentration using the bicinchoninic acid (BCA) method (Walker, 1994). We separated the protein extracts (20 µg each) using 12% SDS-PAGE and transferred the samples to a polyvinylidene fluoride (PVDF) membrane (Millipore, Massachusetts, USA) using the Mini Trans-Blot electrophoretic transfer system (Bio-Rad). We blocked membranes with 5% non-fat milk (diluted with PBS containing 0.1% Tween-20; PBST) for 3 h at room temperature, before incubating with the prepared anti-Ap-serpin-1 polyclonal antibody for 12 h at 4°C. Finally, we incubated the membranes with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma) for 1 h at room temperature. We detected the immunoblot signal using HRP-DAB Detection Kit (Tiangen, Beijing, China).

Expression analysis of Ap-serpin-1 induced by microorganisms

We divided the 5th larvae of *A. pernyi* into four groups; each group contained 18 larvae. We injected each larva with the heat-inactivated gram-negative bacterium (*E. coli*, 8×10^6 cfu), gram-positive bacterium (*Micrococcus luteus* 8×10^6 cfu), fungus (*Beauveria bassiana* 8×10^6 cfu) and virus (nuclear polyhedrosis virus, NPV; 8×10^6 PIBs), which we diluted in sterilized PBS. We used the larvae injected with sterilized PBS as the negative control. As the fat body and hemolymph are involved in the immune defense mechanism (Feng *et al.*, 2011), we chose to analyze how Ap-serpin-1 expression was influenced by microorganism challenges in these tissues. We collected fat bodies and hemolymph from individual samples after 1.5, 3, 6, 12, 24 and 48 h. We collected the tissues of three larvae per sample and repeated the biological sampling three times. We determined the expression levels of Ap-serpin-1 by qRT-PCR as described above.

Result

Molecular characteristic of full-length Ap-serpin-1 cDNA

The complete cDNA sequence and deduced amino acid sequence analysis revealed that *Ap-serpin*-1 contains a putative conserved domain of the serpin superfamily. Full-length cDNA of *Ap-serpin-1* consists of 837 bp that contains a 783 bp ORF, encoding a predicted 279 amino acid residue protein. The protein contains a signal peptide of 18 amino acid residues (MKITVCIFALAAMTLANG). The calculated molecular weight of the protein is 30.8 kDa with an isoelectric point (pI) of 5.24. The Ap-serpin-1 protein shared greatest similarities with the serpin from *Antheraea mylitta* (fig. 2).

Expression and purification of the recombinant Ap-serpin-1 protein

The Ap-serpin-1 protein was expressed in the *pET-28a* (+) vector in *E. coli* (Transetta DE3) cells. SDS-PAGE analysis showed that recombinant Ap-serpin-1 protein was successfully expressed and the expression was not influenced by different IPTG concentrations. Moreover, purified proteins appeared as a single band on the SDS-PAGE gel (fig. 3a). The apparent molecular weight was 32 kDa, which was little higher than the predicted molecular weight (30.8 kDa). The immunoblot assay using the anti-FLAG tag antibody confirmed the presence of recombinant Ap-serpin-1 protein (fig. 3b).

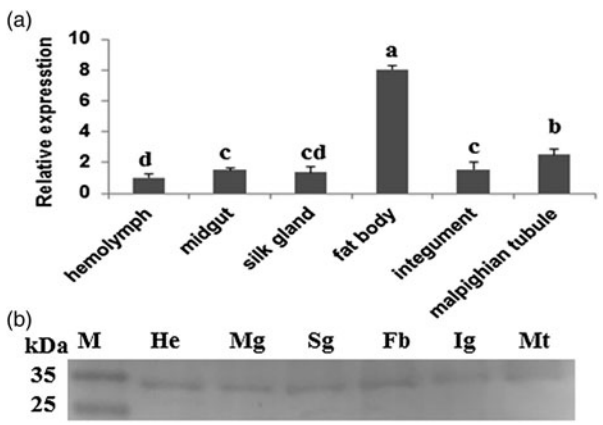

Fig. 4. Tissue distribution of Ap-serpin-1 in the 5th instar larvae of *A. pernyi*. (a) Analysis of serpin-1 mRNA expression in tissues of larvae using quantitative real-time polymerase chain reaction (qRT-PCR). (b) Analysis of Ap-serpin-1 protein expression in tissues of larvae by western blotting. Ap-serpin-1 was detected using an anti-Ap-serpin-1 rabbit polyclonal antibody.

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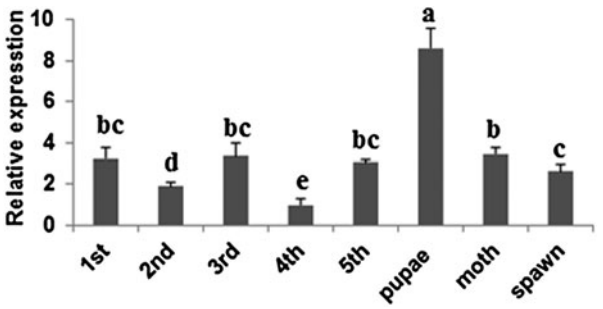

Fig. 5. Expression levels of Ap-serpin-1 during developmental stages. The sequence of period are 1st, 2nd, 3rd, 4th, 5th, pupae, moth and spawn.

Tissue distribution and developmental expression of Ap-serpin-1

We performed qRT-PCR to determine the expression levels of Ap-serpin-1 in different tissues and developmental stages. The analysis revealed that Ap-serpin-1 was expressed ubiquitously in all examined tissues, with higher expression levels found in fat bodies and least expression observed in hemolymph (figs 4a, 4b) Our qRT-PCR and western blotting results demonstrate that Ap-serpin-1 mRNA expression profile is consistent with the protein content profiles. Our qRT-PCR analysis of Ap-serpin-1 expression levels at different developmental stages of *A. perenyi*, indicated that Ap-serpin-1 expression was greatest at the pupal stage and lowest at the 4th larval stage. We recorded insignificant variations at other developmental stages (fig. 5).

Induced expression patterns of Ap-serpin-1 in the fat body and hemolymph

We explored the induced expression profiles of Ap-serpin-1 when infected with *E. coli*, *M. luteus*, NPV and *B. bassiana*. When the fat body from *A. pernyi* larvae were treated with *B. bassiana*, Ap-serpin-1 expression was significantly upregulated from 1.5 to 12 h, and peaked from 24 to 48 h compared with the control group (fig. 6a). When the larval fat bodies were treated with NPV and *M. luteus*, *Ap-serpin-1* expression was significantly upregulated from 3to 48 h, respectively (figs 6b, 6c). Treatment with *E.coli* enhanced Ap-serpin-1 expression compared with the control group. Moreover, expression levels remained unchanged as a function of induction times (fig. 6d).

We also determined expression levels of Ap-serpin-1 in hemolymph following various biotic stresses using qRT-PCR. After *B. bassiana* treatment, Ap-serpin-1 expression was significantly upregulated at 24 to 48 h compared with the control group (fig. 7a). When the larvae were treated with *E. coli*, Ap-serpin-1 expression was upregulated, which gradually decreased from 1.5 to 48 h (fig. 7b). Upon treatment with *M. luteus*, Ap-serpin-1 transcription was only subtly upregulated at 1.5 to 12 h, and became strongly upregulated at 24 to 48 h (fig. 7c). Likewise, at 12 h, NPV treatment did not significantly upregulate Ap-serpin-1 expression, while, in other time period, we observed a significant increase (fig. 7d).

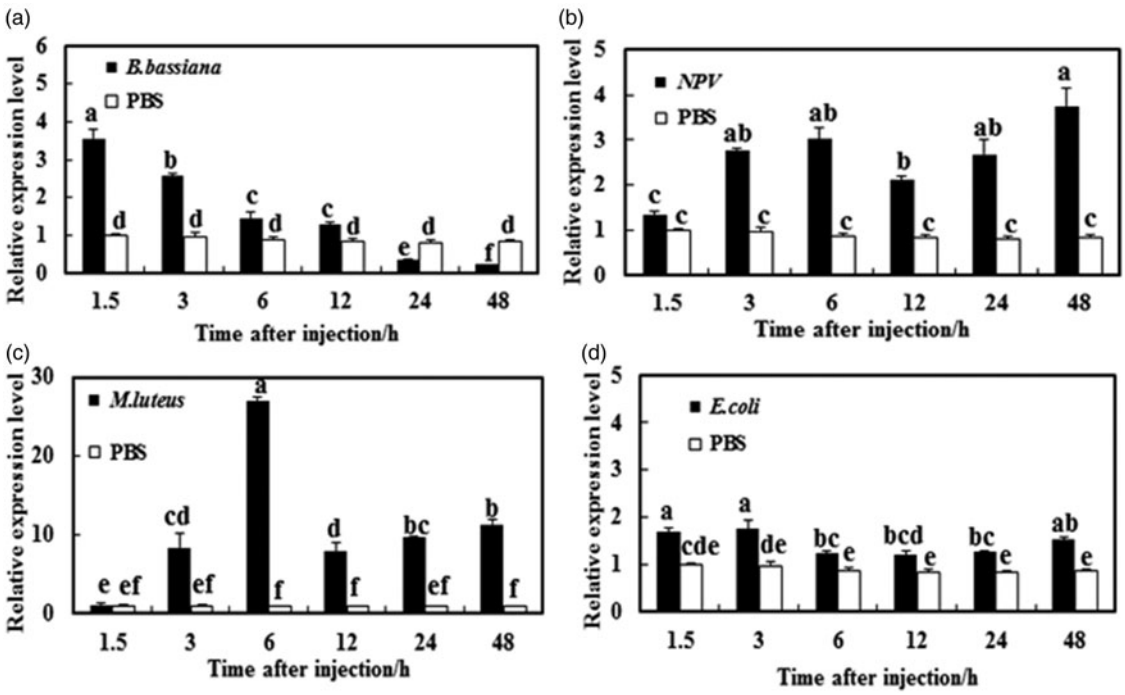

Fig. 6. Expression profiles of Ap-serpin-1 in the fat body of the 5th instar larvae after microorganism challenges. *Ap-serpin-1* mRNA in the PBS-injected fat body was designated as the calibrator. (a–c and d) represent mRNA transcript level of *Ap-serpin-1* in the fat body after challenges with *B. bassiana*, *NPV*, *M. luteus* and *E. coli*, respectively. Bars represent mean ± S.E. ($n = 3$). Bars labeled with different letters are significantly different ($P < 0.05$).

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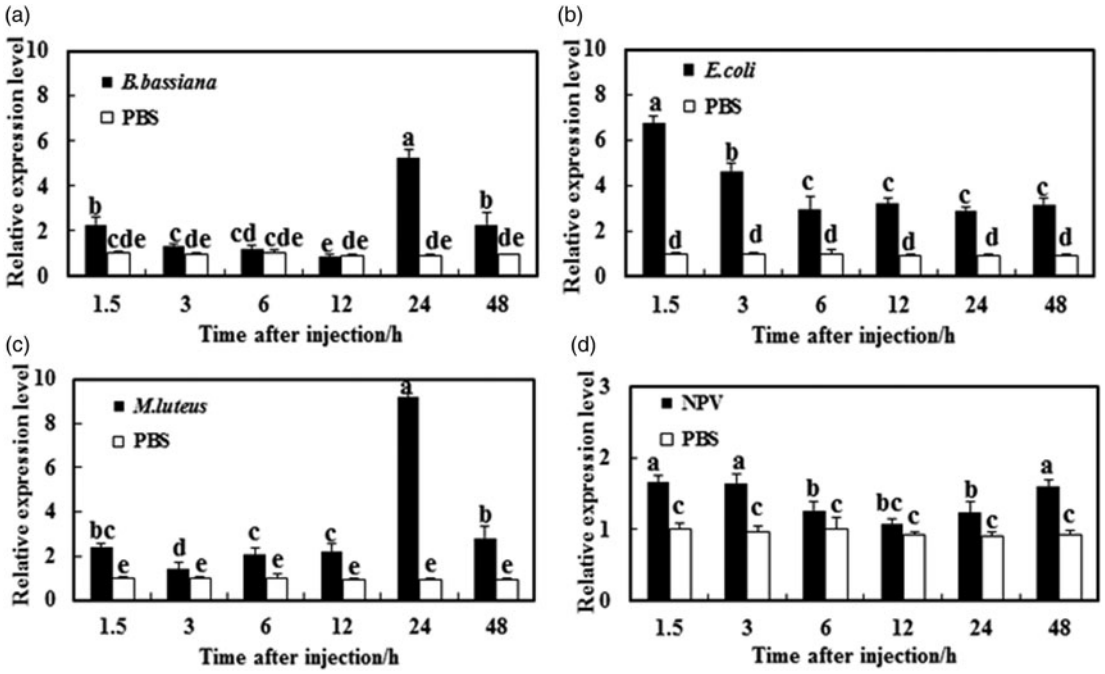

Fig. 7. Expression profiles of Ap-serpin-1 mRNA in the hemolymph of the 5th instar larvae of *A. pernyi*. *Ap-serpin-1* mRNA in the PBS-injected hemolymph was designated as the calibrator. A, B, C and D represent mRNA transcript level of *Ap-serpin-1* in hemolymph after challenge with *B. bassiana*, *E. coli*, *M. luteus* and nuclear polyhedrosis virus (NPV), respectively. Bars represent mean ± S.E. ($n = 3$). Bars labeled with different letters are significantly different ($P < 0.05$).

Discussion

Serpins are widely distributed in eukaryotes and considered to be involved in the regulation of several protease-mediated biological processes such as the immune response, metamorphosis and embryogenesis (Jiang & Kanost, 1997; De Gregorio *et al*., 2002; Zhao *et al*., 2012). In the present study, we identified a serpin-1 gene from *A. pernyi* that encodes a protein with a molecular weight of 30.8 kDa. Phylogenetic analysis revealed that the Ap-serpin-1 was highly homologous to *A. mylitta* serpin-1, which contains the conserved signature of the serpin superfamily.

Our qRT-PCR analysis showed that Ap-serpin-1 was expressed in all examined tissues, with the highest expression levels in the fat body. As far as the developmental stages were concerned, higher expression levels were detected in the pupae. Zheng *et al*., documented higher expression level of serpin-1 in the fat body of *Choristoneura fumiferana*, which was highly expressed during the intermolt phase compared with molting phase (Zheng *et al*., 2009). *M. sexta* serpin-1 was also reported higher larval fat body, which disappeared abruptly at the molting and wandering stages (Kanost *et al*., 1995). Similarly, serpins-1b/1c are expressed predominantly in the fat body and subsequently exported to the hemolymph in *Mamestracon figurate* (Chamankhah *et al*., 2003). It is reported that after bacterial challenges, serpin-1J expression in *M. sexta* decreases the production of antimicrobial proteins like cecropin, attacin and hemolin via the Toll pathway response (An *et al*., 2011). According to our results, Ap-serpin-1 expression in the fat body and hemolymph was significantly upregulated following microorganism challenges, and the expression patterns vary depending on the type of pathogens (*E. coli*, *B. bassiana*, *M. luteus and* NPV), which may be related to the difference in the signaling pathways involved in immune processes of *A. pernyi*.

Altogether, our results suggest that Ap-serpin-1 is involved in the immune response against pathogens. Although we investigated the expression level and patterns of Ap-serpin-1 in *A. pernyi*, the mechanisms involved in the interaction between Ap-serpin-1 and its effectors and its signaling pathways remain unclear. Further studies are needed to evaluate these molecular and signaling mechanisms in order to provide a clearer understanding of the interactions between Ap-serpin-1 and its effectors.

Acknowledgements

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References

Abraham, E.G., Pinto, S.B., Ghosh, A., Vanlandingham, D.L., Budd, A., Higgs, S., Kafatos, F.C., Jacobs-Lorena, M. & Michel, K. (2005) An immune-responsive serpin, SRPN6, mediates mosquito defense against malaria parasites. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 16327–16332.

https://doi.org/10.1017/S000748531700013X Published online by Cambridge University Press

An, C., Ragan, E.J. & Kanost, M.R. (2011) Serpin-1 splicing isoform J inhibits the proSpatzle-activating proteinase HP8 to regulate expression of antimicrobial hemolymph proteins in *Manduca sexta*. *Developmental & Comparitive Immunology* **35**, 135–141.

Bilang Liu, X.Q., Han, Q., Jia, L., Xiang, Z. & He, N. (2012) A silkworm hemolymph protein is a prophenoloxidase activation blocker. *American Journal of Molecular Biology* **2**, 332–340.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

Buss, H., Chan, T.P., Sluis, K.B., Domigan, N.M. & Winterbourn, C.C. (1997) Protein carbonyl measurement by a sensitive ELISA method. *Free Radical Biology & Medicine* **23**, 361–366.

Chamankhah, M., Braun, L., Visal-Shah, S., O'Grady, M., Baldwin, D., Shi, X., Hemmingsen, S.M., Alting-Mees, M. & Hegedus, D.D. (2003) Mamestra configurata serpin-1 homologues: cloning, localization and developmental regulation. *Insect Biochemistry and Molecular Biology* **33**, 355–369.

De Gregorio, E., Han, S.J., Lee, W.J., Baek, M.J., Osaki, T., Kawabata, S., Lee, B.L., Iwanaga, S., Lemaitre, B. & Brey, P. T. (2002) An immune-responsive Serpin regulates the melanization cascade in Drosophila. *Developmental Cell* **3**, 581–592.

Feng, C., Huang, J., Song, Q., Stanley, D., Lu, W., Zhang, Y. & Huang, Y. (2011) Parasitization by *Macrocentrus cingulum* (Hymenoptera: Braconidae) influences expression of prophenoloxidase in Asian corn borer *Ostrinia furnacalis*. *Archieves of Insect Biochemistry and Physiology* **77**, 99–117.

Gettins, P.G.W. (2002) Serpin structure, mechanism, and function. *Chemical Reviews* **102**, 4751–4803.

Harlow, E. & Lane, D. (1999) *Using Antibodies: a Laboratory Manual: Portable Protocol no. i.*, Cold Spring Harbor Laboratory Press.

Irving, J.A., Pike, R.N., Lesk, A.M. & Whisstock, J.C. (2000) Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. *Genome Research* **10**, 1845–1864.

Jiang, H.B. (2008) The biochemical basis of antimicrobial responses in *Manduca sexta*. *Insect Science* **15**, 53–66.

Jiang, H. & Kanost, M.R. (1997) Characterization and functional analysis of 12 naturally occurring reactive site variants of serpin-1 from Manduca sexta. *Journal of Biological Chemistry* **272**, 1082–1087.

Kanost, M.R. (1999) Serine proteinase inhibitors in arthropod immunity. *Developmental & Comparitive Immunology* **23**, 291–301.

Kanost, M.R., Prasad, S.V., Huang, Y. & Willott, E. (1995) Regulation of serpin gene-1 in *Manduca sexta*. *Insect Biochemistry and Molecular Biology* **25**, 285–291.

Law, R.H., Zhang, Q., McGowan, S., Buckle, A.M., Silverman, G. A., Wong, W., Rosado, C.J., Langendorf, C.G., Pike, R.N., Bird, P.I. & Whisstock, J.C. (2006) An overview of the serpin superfamily. *Genome Biology* **7**, 216.

Liu, D.R., Wang, L., Yang, L., Qian, C., Wei, G.Q., Dai, L.S., Li, J., Zhu, B.J. & Liu, C.L. (2015) Serpin-15 from *Bombyx mori* inhibits prophenoloxidase activation and expression of antimicrobial peptides. *Developmental & Comparitive Immunology* **51**, 22–28.

Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)) Method. *Methods* **25**, 402–408.

Sasaki, T. & Kobayashi, K. (1984) Isolation of two novel proteinase inhibitors from hemolymph of silkworm larva, *Bombyx mori*. Comparison with human serum proteinase inhibitors. *Journal of Biochemistry* **95**, 1009–1017.

Sun, Y., Wang, L., Qian, C., Dai, L.S., Li, J., Zhang, C.F., Zhu, B.J. & Liu, C.L. (2015) Molecular cloning and expression analysis of a hemolin-like molecule from *Antheraea pernyi*. *International Immunopharmacology* **26**, 65–71.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731–2739.

Tang, H., Kambris, Z., Lemaitre, B. & Hashimoto, C. (2008) A serpin that regulates immune melanization in the respiratory system of Drosophila. *Developmental Cell* **15**, 617–626.

Tong, Y., Jiang, H. & Kanost, M.R. (2005) Identification of plasma proteases inhibited by *Manduca sexta* serpin-4 and serpin-5 and their association with components of the prophenol oxidase activation pathway. *Journal of Biological Chemistry* **280**, 14932–14942.

Walker, J.M. (1994) The bicinchoninic acid (BCA) assay for protein quantitation. *Methods in Molecular Biology* **32**, 5–8.

Yamasaki, M., Sendall, T.J., Harris, L.E., Lewis, G.M. & Huntington, J.A. (2010) Loop-sheet mechanism of serpin polymerization tested by reactive center loop mutations. *Journal of Biological Chemistry* **285**, 30752–30758.

Zhang, C.F., Dai, L.S., Wang, L., Qian, C., Wei, G.Q., Li, J., Zhu, B.J. & Liu, C.L. (2015) Eicosanoids mediate sHSP 20.8 gene response to biotic stress in larvae of the Chinese oak silkworm *Antheraea pernyi*. *Gene* **562**, 32–39.

Zhang, B., Wu, T.Y., Tang, X.W., Zhang, S.Y., Xu, Q.W., Zhao, Y., Wang, Y.J. & Feng, C.J. (2016) Cloning, expression and characterization of *Ostrinia furnacal*is serpin1, a regulator of the prophenoloxidase activation system. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **192**, 9–20.

Zhao, P., Dong, Z., Duan, J., Wang, G., Wang, L., Li, Y., Xiang, Z. & Xia, Q. (2012) Genome-wide identification and immune response analysis of serine protease inhibitor genes in the silkworm, *Bombyx mori*. *PLoS ONE* **7**, e31168.

Zheng, Y.P., He, W.Y., Beliveau, C., Nisole, A., Stewart, D., Zheng, S.C., Doucet, D., Cusson, M. & Feng, Q.L. (2009) Cloning, expression and characterization of four serpin-1 cDNA variants from the spruce budworm, *Choristoneura fumiferana*. *Comparitive Biochemistry and Physiology B Biochemical and Molecular Biology* **154**, 165–173.

Zou, Z., Picheng, Z., Weng, H., Mita, K. & Jiang, H. (2009) A comparative analysis of serpin genes in the silkworm genome. *Genomics* **93**, 367–375.