The initial analysis of a serine proteinase gene (*AccSp10*) from *Apis cerana cerana*: possible involvement in pupal development, innate immunity and abiotic stress responses

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Abstract Serine proteinases play important roles in innate immunity and insect development. We isolated a serine proteinase gene, designated *AccSp10*, from the Chinese honeybees (*Apis cerana cerana*). RT-qPCR and a Western blot analysis at different pupal development stages indicated that *AccSp10* might be involved in melanin formation in pupae and promote pupal development. In adult workers, the expression of *AccSp10* was upregulated by treatments mimicking harmful environments such as the presence of *Bacillus bombysepticus*, different temperatures (4, 24 and 42 °C), HgCl₂, H₂O₂ and paraquat; the exception was treatment with VC (vitamin C), which did not upregulate *AccSp10* expression. Western blot confirmed the results. A disc diffusion assay indicated that recombinant *AccSp10* accelerated *E. coli* cell death during stimulation with harmful substances (HgCl₂, paraquat and cumene hydroperoxide). These findings suggest that *AccSp10* may be involved in the pupal development of Chinese honeybees and protection against microorganisms and abiotic harms.

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Introduction

Insects are the most widely distributed species in the world, often living in environments with many pathogens (for example, fungi and Gram-negative bacteria) and various unfavourable environmental stressors (extreme temperature, heavy metals, pesticides), which can cause serious oxidative damage (Halliwell and Gutteridge 1989; Yao et al. 2013). All of these factors pose a threat to the lives of insects, including the Chinese honeybees (*Apis cerana cerana*), which is an important species in maintaining the balance of regional ecologies in China.

When pathogens enter insects, insects can initiate a type of humoral immunity, which is an important innate immune response involving serine proteinases that includes coagulation, melanisation and Toll pathway signalling (Muta and Iwanaga 1996; Iwanaga et al. 1998; Jiang et al. 2010). These serine proteinase cascade pathways can rapidly amplify responses to infection and stimulate pathogen killing (An et al. 2009). The coagulation mechanism is well known based on studies in horseshoe crab; plasmozyme is activated by serine proteinase cascade reactions, leading to the coagulation of haemolymph for pathogen clearance (Muta and Iwanaga 1996; Iwanaga et al. 1998). In the melanisation pathway, after pathogen-associated molecular patterns such as fungal β -1, 3-glucan or bacterial peptidoglycan are recognized by pattern recognition proteins in insects (Gupta et al. 2005; Hughes 2012), a series of serine proteinases in haemolymph are activated, which leads to proPO-activating proteinase (PAP) activation and the transformation of proPO (prophenoloxidase) into PO

(phenoloxidase) (Gorman et al. 2007; Wang and Jiang 2007). PO is the key proteinase that catalyses the formation of melanin. This pathway plays an important role in microbe killing and wound healing (Mavrouli et al. 2005; Jiang et al. 2010). The Toll molecule was originally shown to be a type I transmembrane receptor that controls dorsal-ventral patterning of the *Drosophila* embryo (Anderson et al. 1985a, b; Hashimoto et al. 1988) and later was identified to be involved in host resistance against pathogens (Lemaitre et al. 1996; Ligoxygakis et al. 2002; Michel et al. 2001). Many serine proteinases with clip-domains have been found in *Drosophila*, *Manduca sexta* and *Bombyx mori*, which participate in Toll pathway activation and stimulate the synthesis of antimicrobial peptides (metchnikowins, thanatin, magainin) (Kim et al. 2008; An et al. 2009; Roh et al. 2009; Ligoxygakis et al. 2002; Kambris et al. 2006; Bulet et al. 1999; Lehrer and Ganz 1999).

Additional studies have found that extracellular serine proteases are required for dorsal-ventral growth of the *Drosophila* embryo (Belvin and Anderson 1996; Trapasso and Simpson 1998). Ahola et al. (2015) reported that the serine protease family was associated with larval growth rate, development time and pupal weight in the Glanville fritillary butterfly. In mammalian preimplantation embryos, a serine protease (hepsin) was also found to be expressed (Vu et al. 1997). These imply that serine proteases might play a role in the development of various organisms.

These serine proteinases that participate in immunity and insect development are conserved proteinases in terms of their structure, which contains a conservative catalytic triad with the His, Asp and Ser residues (Carter and Wells 1988; Polgár 2005; Jiang and Kanost 2000). Additionally, many serine proteinases have clip-domains at their amino terminus, but little is known about the functions of clip-domains at present (Zou et al. 2006; Ross et al. 2003; Veillard et al. 2015). Serine proteinases exist in haemolymph in the form of zymogens, which are cut and activated at specific sites and then activate downstream proteins, when pathogens invade insects (James and Sielecki 1986; Jiang and Kanost 2000, 2010). At present, functional serine proteinases have been found in *Bombyx mori*, *Drosophila melanogaster*, *Anopheles gambiae*, *Tenebrio molitor*, *Holotrichia diomphalia* and *Manduca sexta*, particularly in *Drosophila melanogaster* and *Manduca sexta* (An et al. 2011; Gorman et al. 2007; An et al. 2009). However, the functions of most clip-domain proteases are unknown, even in well-studied insect species.

All of the previous data indicate that serine proteinases play a crucial role in innate immunity and insect development. However, the melanisation reaction pathway that these enzymes involved has not understood well (Tang et al. 2006; An et al. 2009), particularly in the Chinese honeybees (*A. cerana cerana*). Furthermore, little is known about the functions of serine proteinases when

insects are subjected to abiotic stresses. Thus, the study of serine proteinases in the Chinese honeybees (*A. cerana cerana*) is necessary. Here, we identified a gene (*AccSp10*) from the Chinese honeybees (*A. cerana cerana*) for the first time and predicted that it is a serine proteinase gene that may be involved in Chinese honeybee development and protection against microorganisms and environmental harms.

Materials and methods

Specimens

Insects used in the following experiments were Chinese honeybees (*A. cerana cerana*) from Shandong Agricultural University (Taian, China). According to the criteria of Michelette and Soares (1993), the honeybees in the experiments were used at the following stages: eggs; larvae (L1–L6), pupae including prepupae (P0), white eyes (Pw), pink eyes (Pp), brown eyes (Pb) and dark eyes (Pd); and adults (A1, 1-day post-emergence; A15, 15-day post-emergence; and A30, 30-day post-emergence). The whole bodies of eggs, larvae, pupae and 1-day-old adult workers were collected from the hive. The A15 or A30-day-old adult workers were obtained by marking newly emerged bees with paint. These honeybees were bred at a constant humidity (70%) and temperature (34 °C) and under a 24-h dark regimen (Alaux et al. 2010). The whole bodies of bees were flash frozen in liquid nitrogen and stored at −80 °C.

Bacillus bombysepticus was preserved in our laboratory.

Treatments

The 1-day-old post-emergence adult workers were divided into groups ($n = 40$). Groups 1–3 were exposed to 4, 24 and 42 °C, respectively. Groups 4–6 were treated with *Bacillus bombysepticus* ($OD_{600} \approx 100$), $HgCl_2$ (3 mg/mL) and VC (0.02 mg/mL), which was added to food. Group 7 was injected with H_2O_2 (0.5 μ L of a 2 mM dilution) between the first and the second abdominal segments. Paraquat (10 μ M) was daubed on the thoracic notum of worker bees in Group 8. At specific times, specimens were collected and immediately frozen in liquid nitrogen and stored at −80 °C.

Preparation of RNA and cDNA

The extraction of total RNA and complementary DNA (cDNA) synthesis were performed as previously described (Yao et al. 2013).

PCR amplification conditions

Primers are shown in Table 1. The PCR amplification conditions were 10 min at 94 °C, 40 s at 94 °C, 40 s at 47 °C, 1 min 20 s at 72 °C for 35 cycles, 10 min at 72 °C (primers SC1 and SC2 (listed in Table 1)) and 10 min at 94 °C, 40 s at 94 °C, 40 s at 50 °C, 1 min 30 s at 72 °C for 35 cycles, 10 min at 72 °C (primers RP1 and RP2 (listed in Table 1)), respectively.

Cloning of open reading frame of *AccSp10*

The internal fragment of *AccSp10* cDNA was obtained using primers SC1 and SC2, which were designed based on the conserved sequence of *Sp10* from *Apis mellifera*, *Apis dorsata* and *Apis florea*. Subsequently, the PCR product (open reading frame (ORF) without the 5' and 3' cDNA ends) was ligated into the *pEASY-T3* vector and transformed into *Escherichia coli* strain DH5 α for sequencing.

Bioinformatics analysis

The bioinformatics tools (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) at the National Center for Biotechnology Information (NCBI) were used to analyse conserved sequences of *AccSp10*. The homologous sequences of *AccSp10* in other species from NCBI were aligned using the DNAMAN software. The theoretical isoelectric point and molecular mass of the predicted protein were predicted with the online tool PeptideMass (<http://us.expasy.org/tools/peptide>). SWISS-MODEL was used to predict the advanced structure of the *AccSp10* protein. Antibacterial activity loci were predicted using the website <http://aps.unmc.edu/AP/>.

RT-qPCR analysis

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used to identify the expression patterns of the *AccSp10* gene. Total RNA of different samples was extracted separately, and the cDNA was synthesized as described previously (Yao et al. 2013). A 278-bp fragment of *AccSp10* was obtained using primers SQ1 and SQ2 (listed in Table 1). The

housekeeping gene β -actin from *A. cerana cerana* (XM640276) was used as a control gene, because it has been verified as the most stably expressed gene in honeybees (Scharlaken et al. 2008; Zhou et al. 2016). SYBR Premix Ex Taq (TaKaRa, Dalian, China) was used in a 25- μ L volume on a CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA). We have first validated the primers of *AccSp10*. The melting curves had single peak. The amplification efficiency (*E*) and the correlation coefficients (*R*²) were 95.1% and 0.998, respectively. The PCR cycling conditions were as follows: 95 °C for 30 s and then 40 cycles (95 °C for 5 s, 55 °C for 15 s and 72 °C for 15 s). Lastly, there was a melting cycle from 65 to 95 °C. Each sample was repeated in triplicate, and at least three samples were used for each primer pair. The linear relation, amplification efficiency and data analysis were performed using the CFX Manager Software version 1.1. The analysis of the significant differences was conducted using Duncan's multiple range tests with the Statistical Analysis System (SAS) software version 9.1.

Protein expression and purification of recombinant *AccSp10*

The ORF fragment of *AccSp10* cDNA (without signal sequence), amplified using a pair of primers containing BamHI and XhoI (primers RP1 and RP2), was inserted into the expression vector pET-21a (+) and was then transformed into *E. coli* BL21 cells. The cells were cultured in Luria-Bertani (LB) broth with kanamycin at 37 °C until the cell density reached 0.4–0.6 OD₆₀₀. Then, the *AccSp10* expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at 28 °C for 6 h. The bacterial cells were harvested by centrifugation at 5000 $\times$ g for 10 min at 4 °C, resuspended in a loading buffer.

As described previously, the induced protein was collected and then purified using a HisTrap™ FF column (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Twelve percent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to detect the target protein.

Table 1 The primers used in this study

Primers	Primer sequence (5'–3')	Description
SC1	TGAAGGCATGTCTTGATTATC	Synthesizing cDNA primer, forward
SC2	CCTTTGTATTATTAATCTGGCC	Synthesizing cDNA primer, reverse
SQ1	CAATGAATGGATAAGACCCAGTTG	Q-PCR primer, forward
SQ2	GTCCACCACTATCTCCCTG	Q-PCR primer, reverse
β -s	GTTTTCCCATCTATCGTCGG	Standard control primer, forward
β -x	TTTTCTCCATATCATCCCAG	Standard control primer, reverse
RP1	<u>GGATCCT</u> TAGATAAAGGCCGAAGCATG <i>Bam</i> HI	Primer of protein expression, forward
RP2	<u>TCCGAGAT</u> CTGGCCAACTATATTCTC XhoI	Primer of protein expression, reverse

Recombinant AccSp10 protein identification

SDS-PAGE was performed to characterize the recombinant AccSp10 protein. A target band was picked up and further identified by matrix-assisted laser desorption/ionization-time-of-flight tandem mass spectrometry (MALDI-TOF/TOF, ultrafleXtreme™, Bruker, Germany). In brief, the protein sample was digested with trypsin (Promega, Madison, WI) after by DTT reduction and iodine generation acetamide alkylation process. The extracted peptide mixtures were analysed by MALDI-TOF/TOF MS. All peptide spectra were matched with the online Mascot program (<http://www.matrixscience.com>) against the NCBIprot databases. Carbamidomethyl of Cys was chosen as fixed modification; oxidation of Met was chosen as dynamic modification; and two missing tryptic cleavage were allowed.

Antibody preparation and Western blot analysis

The purified recombinant *AccSp10* (200 µg) was mixed with an equal volume of Freund's complete adjuvant (Sigma, USA) and was injected into the New Zealand rabbit. Second injection was the same with first after 2 weeks. Subsequently, three successive injections of 100 µg of protein mixed with an equal volume of Freund's incomplete adjuvant were administered at 2-week intervals. Fourteen days after the final injection, blood was collected and centrifuged at 3000 rpm for 5 min. The supernatant antibodies were stored at -70 °C, for further application.

To analyse the expression of the *AccSp10* gene at the protein level, total protein was extracted from *A. cerana cerana* at four different pupal developmental stages following treatment with microorganisms and at 24 °C. Anti-AccSp10 serum was used as the primary antibody at a 1:400 (v/v) dilution, and peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody at a 1:2000 (v/v) dilution. Anti- β -actin (Sigma, USA) was used as a control. The proteins were detected using a SuperSignal® West Pico Trial Kit (Thermo Science Pierce, IL, USA).

Antibacterial assay of recombinant AccSp10 protein in vitro

A modified disc diffusion assay from Burmeister et al. (2008) was performed. After overexpressing AccSp10 in *E. coli* BL21, the protein was incubated on LB-kanamycin agar plates at 37 °C for 1 h, and filter discs (6-mm diameter) with different concentrations of HgCl₂, paraquat and cumene hydroperoxide were placed on the agar surface for 12 h at 37 °C. Then, the inhibition zones were measured. *E. coli* BL21 cells with empty pET-21a (+) were used as the negative control.

ProPO activation assay

Recombinant proteins were mixed with tissue protein extract from prepupae, which had a low basal PO activity. The mixtures were incubated at room temperature for 10 min. Protein concentration was measured using the BCA Protein Assay Kit (Jiancheng, Nanjing, China). Forty-microliter tissue protein extract with recombinant proteins (30 µg) in 500 µL of phosphate-buffered saline containing protease inhibitors was mixed with 1500 µL of phosphate-buffered saline saturated with L-3,4-dihydroxyphenylalanine (Sigma, USA). After incubation at room temperature for 30 min, the absorbance at 470 nm of the samples was measured. One unit of PO activity was defined as the unit amount of enzyme producing an increase in absorbance (ΔA_{470}) of 0.001 µg/min. Each experiment was repeated at least three times.

Statistical analysis

Statistics were performed using the mean $\pm$ SD from triplicate experiments ($n = 3$). Statistical significance was determined by Duncan's multiple range test using the SAS version 9.1 software program (SAS Institute, Cary, NC, USA). Significance was set at $P < 0.05$.

Results

Characterization of AccSp10

The ORF of *AccSp10* (GenBank accession number: AKT73552) is 1164 bp and encodes a 387-amino-acid polypeptide with a predicted theoretical isoelectric point (pI) of 7.60 and a molecular mass of 43.39 kDa, as shown in Fig. 1a. Alignment of the AccSp10 protein was performed with homologous proteins from other species, specifically *Apis mellifera* Sp10 (XP_001120043.2), *Manduca sexta* HP6 (AAV91004.1), *Drosophila melanogaster* Persephone (NP_573297.1), *Drosophila melanogaster* snake, isoform A (NP_524338.2) and *Drosophila melanogaster* Spatzle-Processing enzyme (NP_651168.1). To understand the relationship between structure and function for the AccSp10 protein, the tertiary structures of AccSp10 and cleaved AccSp10 were predicted; the catalytic triad and clip-domain of AccSp10 are identified in blue, as shown in Fig. 1b, c.

Transcription of AccSp10 and a Western blot analysis at pupal development

The RT-qPCR results showed that the expression of *AccSp10* was low in larvae and highest in the 30-day-old adult workers. In the pupae, the transcription level of *AccSp10* was highest in

Fig. 1 **a** Multiple amino acid sequence alignment of AccSp10. The three conserved regions (II, III and IV) are marked with *black boxes*. The three amino acid residues (His¹⁸³, Asp²³³ and Ser²⁸³) of the catalytic triad of AccSp10 are indicated with *dots*. The protein active site (Leu¹³⁸ and Ile¹³⁹) is indicated with *arrows*. The clip-domain is *underlined*. **b** The tertiary structure of AccSp10. The tertiary structure of AccSp10 was built using homology modelling in SWISS-MODEL. The catalytic triad of AccSp10 (His¹⁸³, Asp²³³ and Ser²⁸³), N-terminus and C-terminus are shown. The structure of the clip-domain is indicated in *blue*. **c** The tertiary structure of cleaved AccSp10. The catalytic triad (His¹⁸³, Asp²³³ and Ser²⁸³) is shown (colour figure online)

the dark eye stage compared to the other pupal stages (Fig. 2a). From white eyes (Pw) to dark eyes (Pd), pupal colour slowly become black, particularly the eyes, indicating that melanisation might be very rich; thus, we selected pupal stages to evaluate the expression of *AccSp10* at the protein level. In the Western blot assay, anti-AccSp10 was used to detect AccSp10. The results indicated that the protein level of AccSp10 increases from white eyes (Pw) to dark eyes (Pd) in the pupae (Fig. 2b).

Transcriptional expression of *AccSp10* and a Western blot analysis under microorganism and abiotic conditions

The RT-qPCR results indicated that the expression of *AccSp10* was upregulated under *Bacillus bombysepticus* and abiotic conditions (4 °C, 24 °C, 42 °C, HgCl₂, H₂O₂ and paraquat) but not under VC treatment (Figs. 3a, b and 4a–c). During the Western blot assay, anti-AccSp10 was used to detect AccSp10. Under microorganism treatment and at 24 °C, AccSp10 protein levels first declined and then rose at different treatment times (Fig. 3c, d).

Overexpression and purification of AccSp10

The ORF fragment of *AccSp10* cDNA (without the signal sequence) was expressed in *E. coli* BL21, and the recombinant AccSp10 protein was purified with HisTrap™ FF columns and is shown in lanes 6 and 7 (Fig. 5). The concentration of the soluble recombinant protein was approximately 1.8 µg/µL.

Recombinant AccSp10 protein identification

The target strip, as shown in Fig. S1A (provided as Supplemental data), was picked up and further identified by MALDI-TOF/TOF MS. The results showed that the protein score (130) was greater than 93 ($P < 0.05$) and significant; the matched protein was serine proteinase 10 (GenBank accession number: AKT73552.1, *Apis cerana cerana*), as shown in Fig. S1B (provided as Supplemental data). The matched peptides were shown in Fig. S1C (provided as Supplemental data). These results indicated that the recombinant protein was the same protein coding by the sequence.

Fig. 2 Expression profiles of *AccSp10* and a Western blot analysis of *AccSp10* at different developmental stages. **a** Transcriptional expression levels of *AccSp10* at different developmental stages: egg, larvae from the first to sixth instars (L1–L6), pupae (*Po* prepupae, *Pw* white eyes, *Pp* pink eyes, *Pb* brown eyes, *Pd* dark eyes) and adults (*A1* 1-day post-emergence, *A15* 15-day post-emergence, *A30* 30-day post-emergence). The data

represent the means $\pm$ SEM ($n = 3$). Letters above the columns indicate significant differences ($P < 0.001$). **b** A Western blot analysis of *AccSp10* at different pupal developmental stages. *Pw* white eyes, *Pp* pink eyes, *Pb* brown eyes, *Pd* dark eyes. The β -actin protein was used as an internal control. All lanes were loaded with equivalent amounts of protein

Antibacterial assay with recombinant *AccSp10* protein

To evaluate the capacity of recombinant *AccSp10* as a serine protease in vitro, a disc diffusion assay was performed. *E. coli* cells with overexpressing *AccSp10* were exposed to HgCl_2 , paraquat and cumene hydroperoxide. The results showed that the death zones of *E. coli* with

overexpressing *AccSp10* were larger in diameter on experimental plates than control plates (Fig. 6).

After culturing the bacteria with *AccSp10* 6, 7 and 8 h, the absorbance of bacteria was measured at 600 nm, respectively; results showed that the absorbance of bacteria with *AccSp10* was less than the control (without *AccSp10*) (Fig. 7).

Fig. 3 The expression profiles of *AccSp10* and the Western blot analysis of *AccSp10* with *Bacillus bombysepticus* ($\text{OD}_{600} \approx 100$) and 24 °C treatments. **a**, **b** Transcriptional expression levels of *AccSp10* with *Bacillus bombysepticus* ($\text{OD}_{600} \approx 100$) and 24 °C treatments. The data are the means $\pm$ SEM ($n = 3$). Letters above the columns indicate

significant differences ($P < 0.001$). **c**, **d** The Western blot analysis of *AccSp10* with *Bacillus bombysepticus* ($\text{OD}_{600} \approx 100$) and 24 °C treatments. The β -actin protein was used as an internal control. All lanes (each condition) were loaded with equivalent amounts of protein

Fig. 4 The expression profiles of *AccSp10* under different abiotic environmental conditions. These conditions were temperature (a), paraquat (b(c)), HgCl₂ (b(d)), H₂O₂ (c(e)) and VC (c(f)). The β -actin gene was used as an internal control. The data represent the means $\pm$ SEM ($n = 3$). Letters above the columns indicate significant differences ($P < 0.001$)

ProPO activation assay

The PO activity of tissue protein extract with recombinant *AccSp10* was higher than the control (Fig. 8).

Discussion

In insects, serine proteinases with serine in their active centres are likely to be involved in development and innate immunity physiology (Krem and Di Cera 2002; Rawlings and Barrett 1993; Zou et al. 2006). According to reports, many serine proteinases with clip-domains were shown to participate in melanism reactions to help clear pathogens and initiate the Toll pathway, in which antimicrobial peptides are produced (An et al. 2009; Jiang et al. 2010; Aggarwal and Silverman

Fig. 5 An SDS-PAGE analysis of the expression and purification of recombinant *AccSp10* protein. Lanes 1 and 2, non-induced and induced overexpression of recombinant *AccSp10* protein (10 μ L, respectively); lane 3, protein molecular weight marker; lanes 4 and 5, suspension of sonicated recombinant *AccSp10* protein (10 μ L, respectively); lanes 6 and 7, purified recombinant *AccSp10* protein (18 and 10.8 μ g, respectively)

2008; Gabriella et al. 2011). These studies were also performed in *Manduca sexta* and *Drosophila melanogaster*. In this study, a serine proteinase gene from *A. cerana cerana*, designated *AccSp10*, was cloned and preliminarily analysed under simulated environments for the first time. The results suggest that *AccSp10* takes part in stress responses and likely plays a part in honeybee development and protection against harmful damage.

A sequence analysis indicates that *AccSp10* is a typical serine proteinase with three highly conserved sequences (TAAHC, DIAL and GDSGGP) in which the residues His¹⁸³, Asp²³³ and Ser²⁸³ form a catalytic triad that is essential for a serine protease to maintain catalytic functions (Jiang and Kanost 2000). The protein active site (Leu¹³⁸ and Ile¹³⁹) was predicted from which *AccSp10* was cleaved and activated with a surplus molecular mass of 27.78 kDa. After *AccSp10* is activated, it may then activate downstream interacting proteins. These features of serine proteinases have been identified in many studies (Gupta et al. 2005; Jiang et al. 2010). In addition, a clip-domain (residues from Cys²⁵ to Pro⁷⁰) was predicted in the *AccSp10* sequence. Studies have indicated that serine proteinases with clip-domains play a significant role in embryonic development and immune responses (An et al. 2009; Belvin and Anderson 1996; Iwanaga et al. 1998; Kanost et al. 2004; Ligoxygakis et al. 2002). Based on previous studies, it appears that *AccSp10* is a typical serine protease with a clip-domain and might possess the same functions as other serine proteases such as *MsHp6* or *DrsPersephone* (An et al. 2009; Ming et al. 2014).

Melanin synthesis and deposition in the cuticle occur at specific times during insect development. Phenoloxidase is an important enzyme in the biosynthesis of melanin and exists as an inactive precursor, prophenoloxidase. When phenoloxidase is activated by a cascade of serine proteases, it can catalyse

Fig. 6 Antibacterial activity assay with recombinant AccSp10 protein employing the disc diffusion method. LB agar plates were inoculated with 5×10^8 cells. *E. coli* BL21 cells transfected with empty pET-21a (+) vector were used as controls. The filter discs labelled 1 represent the negative controls. Filter discs labelled 2, 3, 4 and 5 represent different concentrations of HgCl₂ (a, d), paraquat (b, e) and cumene hydroperoxide (c, f). The diameters of the death zones were measured. The data shown are the means $\pm$ SEM ($n = 3$)

reactions that lead to melanin and cuticle sclerotization (Andersen 1985; Hiruma and Riddiford 1988; Yoshida and Ashida 1986). Bitondi et al. (1998) reported that cuticular pigmentation of *Apis mellifera* correlated with phenoloxidase activation. The process of cuticular pigmentation begins at the pupal phase in *Apis mellifera* (Rembold 1987). This study indicated that serine proteases activate phenoloxidase and give rise to melanin during pupal development. To further probe the function of *AccSp10* in development, the expression pattern of the *AccSp10* gene was evaluated at different developmental stages. The transcription levels of the *AccSp10* gene were measured using qPCR and exhibited large differences at all experimental stages, as shown in Fig. 2a. The transcription expression of *AccSp10* was highest in the dark eyes (Pd) stage than

Fig. 7 The AccSp10 inhibition for the growth of *Escherichia coli* BL21 cells. The inhibition for the growth of *E. coli* BL21 cells. The absorbance was measured after 6, 7 and 8 h, respectively. *E. coli* BL21 cells transfected with empty pET-21a (+) vector were used as controls

the other pupal stages. The Western blot assay showed that the *AccSp10* protein was upregulated gradually with pupal growth, and there was greater accumulation at the brown eyes (Pb) and dark eyes (Pd) stages. Pw (white eyed) is the junction from larva to pupae, which is a period during which cells become highly differentiated, and many organs tend to formation, for example, formation of the head, thorax, abdomen and legs; the result of *AccSp10* for RT-qPCR is not in line with WB at Pw stage, as shown in Fig. 2, suggesting that the *AccSp10* may quickly participate in other development processes besides melanisation reaction or the translator process of *AccSp10* may be regulated after transcription. These results indicated

Fig. 8 The proPO activation assay. PO activity was measured using dopamine as a substrate. The tissue protein extract without recombinant *AccSp10* was used as the control. The data represent the means $\pm$ SEM ($n = 3$). Asterisks indicate a significant difference from the control at $P < 0.001$

that *AccSp10* was likely involved in the production of melanin and promoted the development of pupae, especially changes in body surface colour, which might facilitate improved insect immunity.

Studies have shown that serine proteinases can initiate cascade reactions to resist pathogen invasion and promote wound healing (Muta and Iwanaga 1996; Lavine and Strand 2001; Jiang et al. 2010). To further understand the functions of *AccSp10*, external environment conditions were imitated. In this study, we found that the transcriptional expression of *AccSp10* was upregulated when adult workers were treated with *Bacillus bombysepticus*, as shown in Fig. 3a. Western blot showed that *AccSp10* protein levels first declined and then rose, as shown in Fig. 3c. This suggested that *AccSp10* may take part in the elimination of *Bacillus bombysepticus* via lash-up and stress reactions. When accepting the external stimulus, organisms could occur lash-up and stress reactions (lash-up reactions showed quick reflexes, but stress reactions slow), *AccSp10* expressed less in the early during lash-up reactions because of instinct, for example, during bacterial immune challenge at 3 h, at the same time *AccSp10* protein was largely used to resist abiotic stresses; then, with the emergence of stress reactions, the expression of *AccSp10* was greatly upregulated by hormone; so the transcription of *AccSp10* was lower at 3 h and higher at 24 h, the result of Western blot was gradually higher from 3 to 24 h and the protein was less at 3 h than the control because of being consumed. This phenomenon could be observed under other stresses. The expression of *AccSp10* was also upregulated at extreme temperatures (4, 24 and 42 °C) and under harmful abiotic conditions at different time points (treated with HgCl_2 , H_2O_2 and paraquat) (Fig. 4a–c(e)). An and Choi (2010) found that temperature is an abiotic environmental factor that induces physiological changes in organisms. When the temperature is higher than a certain range, it can induce damage in insects, including water imbalance, changes in the ion concentration in cells, membrane destruction and structural changes to DNA and proteins (Yoder and Denlinger 1991; Hallman and Denlinger 1998; Walter et al. 1990; Jaenicke 1991; Greenspan et al. 1980). Cold injury globally influenced biological structures and processes and was a primary cause of insect death (Hodkova and Hodek 2004; Košťál et al. 2004, 2007). The best survival temperature of Chinese honeybees was approximately 34 °C, so 4, 24 and 42 °C were extreme temperatures. When adult workers were exposed to extreme temperatures (4, 24 and 42 °C), *AccSp10* mRNA accumulation was greater than controls and higher especially at 24 than 4 and 42 °C (Figs. 3b and 4a). The results indicated that Chinese honeybees had stronger ability to regulate expression of *AccSp10* to pull through abiotic harms at 24 than 4 and 42 °C. Upregulation of the *AccSp10* protein was similarly shown in the Western blot assay at 24 °C (Fig. 3d). These results might imply some other potential role of *AccSp10* in

response to extreme temperatures, suggesting that this gene may play a role in overwintering and oversummering similar with the Hsps (Yang et al. 2016). Li et al. (2005) reported that heavy metal could destroy the activity of the insect antioxidant enzyme system and cause cell damage. H_2O_2 , a typical oxidant, can cause oxidative damage, resulting in cell death (Goldshmit et al. 2001). Paraquat, one of the most widely employed herbicides in the world, can produce super oxygen anion free radicals and damage the chloroplast membranes of plant. The upregulated *AccSp10* at different times after exposure to extreme temperatures, HgCl_2 , H_2O_2 and paraquat suggested that this gene play a potential role in environmental adaptation. However, we also found that *AccSp10* was downregulated when honeybees were treated with VC (Fig. 4c(f)). Etlik et al. (1997) reported that vitamins protected red blood cells from lipoperoxidation induced by sulphur dioxide. This suggested that the workers likely enhance immunity against oxidative damage under these conditions and maintain life through other processes rather than the *AccSp10* pathway. All of the previous data indicate that *AccSp10* might be involved in pathogen clearance, wound healing and protection against microorganisms and abiotic harm.

To further demonstrate the role of *AccSp10* in pathogen elimination, an in vitro disc diffusion assay was performed. The killing zones for *E. coli* overexpressing *AccSp10* were larger in diameter on the experimental plates than on control plates (Fig. 6), indicating that *AccSp10* plays a role in killing *E. coli* cells after activation by different concentrations of HgCl_2 , paraquat and cumene hydroperoxide. In addition, while culturing the bacteria with *AccSp10*, we also found that it took longer for the bacteria with *AccSp10* to reach the same OD_{600} (Fig. 7); above indicated that *AccSp10* might inhibit bacterial growth. The MIC (minimum inhibitory concentration) assay could further measure the antibacterial specificity of recombinant *AccSp10*, but because the recombinant protein we got was easy to form precipitation, we felt regretted extremely that we could not obtain a MIC value of recombinant *AccSp10*. To further explore the antibacterial mechanism exerted by *AccSp10*, the antibacterial activity loci of *AccSp10* were predicted using the website <http://aps.unmc.edu/AP/>. We found that there were two antimicrobial loci (showed in blue and red colours) present in activated *AccSp10*, as shown in Fig. S2a and S2b (provided as Supplemental data). This indicated that *AccSp10* might play an antimicrobial role like other antimicrobial peptides (Bulet et al. 1999). All of the previous data suggest that *AccSp10* is likely involved in the clearance of *E. coli* cells to protect honeybees against bacterial damage. To further verify this view, we performed the proPO activation assay; result showed that tissue protein extract had low basal PO activity that could be activated dramatically by addition of recombinant *AccSp10* (Fig. 8). This powerfully indicated that *AccSp10* could kill pathogen by melanisation pathway.

In conclusion, we identified a gene from *A. cerana cerana*, *AccSp10*; predicted its physiological course, including its participation during different developmental stages and then analysed its functions in the elimination of harmful microorganisms and resistance to abiotic stresses. Furthermore, new studies will test whether *AccSp10* is really involved in the melanism response or Toll pathway signalling to protect honeybees against pathogen damage. Furthermore, this information will provide a foundation for the prevention and cure of honeybee diseases.

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