ISJ 14: 330-339, 2017 ISSN 1824-307X

RESEARCH REPORT

Functional analysis of hemolin gene from silkworm, *Bombyx mori* - immune and development

X Liu, J Li, Q Wang, H Xia, K Chen

Institute of Life Sciences, Jiangsu University, Zhenjiang 212013, P.R. China

Accepted August 31, 2017

Abstract

Hemolin, an immunoglobulin-like protein, is involved in the immunity and development of Lepidoptera, but its exact functions are still unclear. In this work, the quantitative PCR and Western blot analysis were used to study the effects of exogenous substances on hemolin transcription and translation in the silkworm midgut. Also, RNA interference was used to examine the effects of hemolin on wing disc development of silkworm. we found that the transcription and expression of *hemolin* gene could be up-regulated effectively after injection of inactive *Escherichia coli* and cell wall components (LPS and PGN) into silkworm midgut. However, no any changes in transcription and expression of *hemolin* were observed by the injection of BmNPV (*Bombyx mori* nuclear polyhydrosis virus). The protein-protein interaction between hemolin and yippee was verified by Co-immunoprecipitation (Co-IP) in silkworm wing discs. In addition, the silence of *hemolin* gene by RNA interference could lead to adult wing hypoplasia in most hatched moths. Our study provides some useful information regarding the functions of hemolin associated with the immune and developmental mechanisms in silkworm.

Key Words: *Bombyx mori*; immunity; development; hemolin; yippee

Introduction

Hemolin, an immunoglobulin-like protein initially found in lepidopteran insects, is a member of the immunoglobulin superfamily and has been suggested to participate in the immune response to various pathogens, metamorphosis, and the regulation of embryonic development (Schurmann *et al*., 2001). Since it was first discovered in *Hyalophora cecropia* in 1975, the genome sequences for more than 10 species of Lepidoptera, including silkworm (*Bombyx mori*), have been identified based on their structural similarity (Faye *et al*., 1975; Sun *et al*.,1990). More recently, a hemolin-like protein which shares high similarity with insect hemolins, named LvHemolin, has been identified from *Litopenaeus vannamei* and could also play an important role in shrimp innate immune defense against bacterial and viral infections (Zuo *et al.,* 2015).

Corresponding author:
Xiaoyong Liu
Institute of Life Sciences
Jiangsu University
301 Xuefu Road, Zhenjiang 212013, P.R. China
E-mail: liuxiaoyong@ujs.edu.cn

List of abbreviations:
BmNPV: *Bombyx mori* nuclear polyhydrosis virus; PCR: polymerase chain reaction; qPCR: quantitative PCR; RNAi: RNA interference.

Transcription and expression of the *hemolin* gene mainly occur in the insect fat body. Some of them could be secreted into the hemolymph to participate in the immune response. Under normal circumstances, hemolin is present at lower levels in the hemolymph although its expression levels change with the developmental stages of the insect. However, upon infection by bacteria, hemolin titer in the hemolymph increases rapidly, strongly suggesting a functional role for hemolin in humoral immune defense. Hemolins in Lepidoptera own the conservative horseshoe-shaped structure composing immuneglobulin domains, which are similar to *Drosophila* neuroglian cell adhesion molecule (Terenius, 2008). Therefore, they are thought to be the most common type of cell adhesion molecules and regulate the cellular immune response through binding to the cell surface ligand specificity. Hemolin interacts with lipopolysaccharide (LPS) in gram-negative bacteria, lipoteichoic acid in gram-positive bacteria, and β-1,3-glucan on the fungal cell wall (Daffre and Faye, 1997). It exhibits two binding sites for LPS, one for the interaction with lipid A phosphate groups and the other for the interaction with the O-specific antigen and outer-core carbohydrates of LPS (Yu and Kanost, 2002). This is suggestive of its function as a pattern recognition molecule in the cellular immune response.

Hemolin has also been shown to participate in the immune response to viruses in some but not all

species (Terenius *et al*., 2007). For example, it is involved in antiviral resistance in *Bombycoidea*, but not in *Noctuoidea* (Terenius *et al*., 2009). Lepidoptera are susceptible to viral infection only in the larval stage. With the growth and development, they display an increased resistance against viruses and the *hemolin* gene expression is strongly up-regulated accordingly (Kirkpatrick *et al*., 1998). Moreover, growing body evidences suggest a functional role for hemolin in the development of insects. Upon injecting *hemolin*-specific siRNA, the *H. cecropia* pupae could incubate normally, but do not produce fertile eggs after mating (Bettencourt *et al*., 2002). In *Plodia interpunctella*, *hemolin* gene expression is significantly affected by molting hormone regulation before pupation, where the main site of expression is epidermis, and fat body also show some expression as well (Roxstrom-Lindquist *et al*., 2005).

An intracellular protein, yippee, was initially identified from the proteins encoded in a *Drosophila* imaginal disc cDNA library in a yeast interaction trap screen as physically interacting with *H. cecropia* Hemolin (Roxstrom-Lindquist and Faye, 2001). Subsequently, yippee and YPEL (Yippee-like protein) family of proteins, showing extremely high degree of sequence homology, were found in all virtually eukaryotes, including fungi, plants, and animals (Hosono *et al*., 2004, 2010). Yippee from silkworm (BmYippee) has shown to be highly homologous with the *Drosophila* yippee. It encodes 121 amino acid residues with no signal peptide. A tissue specific expression element C/EBP locates in the promoter at the NF-kb sequence. BmYippee is expressed in pupae and eggs and distributed around the mitotic apparatus (Xin *et al*., 2009; Hosono *et al*., 2010). Several lines of evidence suggest that yippee protein plays an important role in eukaryotes that is involved in the cell cycle, cell proliferation and growth (Hosono *et al*., 2004, 2010; Liang *et al*., 2010). However, little is known about the exact function of yippee in development process.

Silkworm is an ideal representative insect for studying immune, development, and metamorphosis in Lepidopterans. Using silkworm as a model organism has numerous advantages, including rich genetic materials, well-studied genetic background, and easy maintenance. Therefore, the silkworm, *Bombyx mori*, was used in this work to investigate the effects of hemolin in immune challenges and on the development of insect. In silkworm, the wing disc, one of the imaginal discs, is a key organ for the development of adult wing. In addition, both yippee and hemolin are present in silkworm. It is important to study the yippee-hemolin interaction in silkworm wing discs that will contribute significantly to the understanding of novel mechanisms involved in development process.

In this paper, xenobiotics and pathogens were used to study the *hemolin* gene expression in the silkworm midgut. Also, the interactions between hemolin and yippee in the wing discs of silkworm were investigated. The inhibition of *hemolin* expression at the pupal stage and its role in the development of wing discs indicate that hemolin is involved in the development of the silkworm wing.

Materials and Methods

Chemicals, cell line and animals

All reagents and chemicals used in this study were purchased from Sigma-Aldrich, Gibco-BRL, and Invitrogen except as otherwise indicated. The strains of *Bombyx mori* named Jingsong × Haoyue were stored in our laboratory, and all larvae were reared with fresh mulberry leaves at 27 °C. Kunming mice and New Zealand white rabbits were provided by Jiangsu University laboratory animal center. BmN cells derived from ovary of *B. mori* and *E. coli* strains (DH5α, TG1, BL21) were kept in our laboratory.

Preparation of antisera against BmHemolin (hemolin from Bombyx mori*) and BmYippee (yippee from* B. mori*)*

Protein sequence of hemolin in *B. mori* was obtained from NCBI database (GenBank: ACQ82817.2) using protein prediction software DNAstar 6.0. The primers were designed using primer design software "Primer Premier 5.0" based on the sequence corresponding to the predicted hydrophilic region. The forward primer was 5′-AGAATTCATGCAAACCAAAGCTTCGGA-3′ and the reverse primer was 5′-ACTCGAGCTATTTATCGTTGGCATCGTC-3′. Protein sequence of Yippee in *B. mori* was also obtained from NCBI database (GenBank: BABH01000166). The primers corresponding to the full-length open reading frame of *Yippee* gene were designed as follows: forward primer, 5′-CGCGGATCCATGGGTAAAATATTTCTTGATC-3′; reverse primer, 5′-CCGCTCGAGCCCTTCCTTGTATCTTTGA-3′. *Hemolin* and *yippee* target fragments were amplified using the Pfu DNA polymerase (TAKALA, Dalian, China) and synthesized primers (Generay, Shanghai, China). The target fragments were cloned into T vector (TAKALA, Dalian, China) for amplification and sequence identification (Sangon Biotech, Shanghai, China). After sequence identification, target fragments for *hemolin* or *yippee* were inserted into the expression vector pET-30a (TAKALA, Dalian, China) for the generation of recombinant plasmids, pET-BmHemAG and pET-BmYipAG, respectively. The recombinant plasmids were transformed into *E. coli* BL21 (DE3) with kanamycin for screening. After IPTG induction, the expressed proteins were subjected to SDS-PAGE and visualized by Coomassie Brilliant Blue staining. The target proteins were cut and dried using a vacuum freezing dryer; then dried gels were grinded to powder for the preparation of antisera. The animal immunization against BmHemolin was performed in Kunming mice using complete freund's adjuvant (Sigma-Aldrich, Shanghai, China). The effects of polyclonal antiserum were tested at the third day after the fourth boosting injection of antigen. Similarly, New Zealand white rabbits were immunized 4 times using BmYippee protein as immunogen. Collected blood serum from mice or rabbits with high sensitivity to target proteins was stored at -80 °C in a cryogenic refrigerator.

Treatment of xenobiotics in BmN cells and silkworm larvae

Treatment of xenobiotic in BmN cells

The BmN cell line, maintained in our laboratory, is derived from the silkworm ovary and has been wildly used for the studies of insect physiology, developmental biology, pathology, molecular biology and so on. After the recovery training, the cells were grown as an adherent culture in TC-100 insect cell culture medium supplemented with 10 % fetal bovine serum (FBS) (Gibco, USA) at 27 °C. The cell culture medium was changed with fresh medium every 3 - 4 days. When the monolayer reached about 80 % confluence, 5 µl of xenobiotic solution was added to the medium in the form of inactivate *E. coli* DH5α, peptidoglycan (PGN), or lipopolysaccharide (LPS) (Sigma-Aldrich, Shanghai, China), respectively. Each treatment group has three replicates. At 24 h after the addition of xenobiotic solution, the adherent cells from 3 flasks of each group were scraped and collected in sterilized EP tubes. After centrifugation at 600 rpm for 1 min, the harvested cell pellets were stored at -80°C till further use.

Treatment of xenobiotics in silkworm larvae

Silkworms were fed with mulberry leaves in a sterile environment. The fifth instar larvae were injected with inactivate *E. coli* (DH5α, about 5×10^7 bacterial cells each silkworm), peptidoglycan (PGN, 5 µl of 1 mg/ml solution), lipopolysaccharide (LPS, 5 µl of 1 mg/ml solution), BmNPV (5 µl of virus, with 1.6×10^4 virus particles/ml), and PBS (5 µl) in separate treatment served as the control group. Each group contained 30 larvae. The wounds were sealed with Vaseline immediately after injection to prevent the loss of injected material and any inadvertent infection. At 9, 24, and 48 h post injection, 10 larvae from each group were placed on a sterile operating table and their midgut tissues were carefully dissected out, rinsed clean with PBS in a 1.5-ml centrifuge tube, and then immediately stored at -80 °C until further analysis.

RNA extraction and quantitative PCR (qPCR)

The stored BmN cell pellets or extracted midgut tissues were taken from -80 °C and grinded in liquid nitrogen. Total RNA was extracted by the TRIzol method (TransGen, Shanghai, China). The quality of extracted RNA was checked by agarose gel electrophoresis and stored at -80 °C. The primers for amplification of silkworm *Hemolin* gene (GenBank: AB115084.1) and *α-tubulin* gene were designed using primer design software "Primer Premier 5.0". For *hemolin* gene, the forward primer was 5′-GTCACTCGGAACTCCCCTA-3′ and the reverse primer was 5′-GACACTCTGCCCAAACCTC-3′. While for *α-tubulin* gene, the forward primer was 5′-GTGTTCAGCCAAACCAAA-3′ and the reverse primer was 5′-TACAGCGGAGTTCAAAGG-3′. The amplification procedure was as follows: Pre-denaturation at 95 °C for 10 min, followed by 25 cycles at 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec. Each sample was tested in triplicate. The relative expression was evaluated following the quantification equation, $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

Western blot analysis

The collected midgut tissues were completely ground into powder in liquid nitrogen. RIPA lysis buffer (strong) supplemented with phenylmethylsulfonate (PMSF) at a final concentration of 10 mM was then added to the ground powder and mixed well by vortexing. The mixture was centrifuged at 13000 rpm for 15 min. The supernatant containing total proteins was collected and protein concentration was determined by the BCA protein assay kit (Pierce). The total proteins from tissues were separated by electrophoresis on SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 5 % w/v nonfat dry milk in TBST buffer for one hour, and then incubated with our prepared polyclonal antiserum (1: 500) for one hour at room temperature. After three 15-min washes with TBST buffer, the membrane was incubated with HRP labeled goat anti-mouse or anti-rabbit IgG (1: 5000) (Beyotime, Shanghai, China) for one hour according to the source of used primary antibody and washed with TBST buffer 3 times. The primary antibody used for reference protein was anti-α-tubulin monoclonal antibody (1:5000). DAB reagent kit (ZSGB-BIO, Beijing, China) was used for signal generation.

Immunoprecipitation and immunodetection

Wing disc tissues from the fifth instar larvae were collected and ground into a powder in liquid nitrogen. Total tissue protein lysate samples were prepared as described above. Immunoprecipitation was performed according to the methods previously described (Golemis, 2002). Briefly, 40 µl of mouse polyclonal antiserum anti-BmHemolin was added into 1 ml of each lysate sample in a sterile 1.5-ml EP tube and incubated on ice for 2 h. In the control test, a mouse monoclonal antibody anti-Vta1 (Novus Biologicals) was used. Then, 30 µl of protein A/G agarose beads (Cwbiotech, Shanghai, China) was added, followed by a slow rotation for overnight at 4 °C. After the incubation, the beads were spun down and washed with RIPA buffer 4 times. The immunoprecipitates were collected and suspended in 2×loading buffer. After boiling at 100 °C for 10 min, the samples were subjected to Western blot analysis using polyclonal antiserum anti-BmYippee. Alternatively, a parallel immnuoprecipitation reaction was performed with anti-BmYippee polyclonal antiserum. The final immunoprecipitates were subjected to Western blot analysis using anti-BmHemolin polyclonal antiserum to detect BmHemolin protein in co-precipitates.

RNA interference of hemolin *gene in silkworm pupae*

Based on the sequence of *hemolin* gene, a BLAST homology search (http://www.ncbi.nlm.nih.gov/BLAST) was performed to avoid off-target effects on other genes or sequences. The siRNAs, synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) as shown in Table 1, were dissolved in diethylpyrocarbonate-treated water at three concentrations (0.4, 0.8, and 1.6 µg/µl H_2O). A 5 µl aliquot of siRNA solution was injected into each pupa in the position of the wing disc using a microliter syringe. Correspondingly, the amount of injected siRNAs was 2, 4,

Table 1 The siRNA fragments of *hemolin* gene for RNA interference

The pairs of Fragment	Sequence (5'-3')	Base Number (bp)
Pair 1 (RNAiBmHem-1)	F: CCGUUAAUUCCGGAGACAATT R: UUGUCUCCGGAAUUAACGGTT	21 21
Pair 2 (RNAiBmHem-2)	F: CCAGCACAAGGGCUACUAATT R: UUAGUAGCCCUUGUGCUGGTT	21 21
Pair 3(RNAiBmHem-3)	F: GCGAUCUGACGUAUCUAUATT R: UAUAGAUACGUCAGAUCGCTT	21 21
Negative control	F: UUGUCCGAUCGUGUCACGUTT R: ACGUGACACGAUCGGACAATT	21 21

and 8 μg per pupa in each group. To avoid leakage of siRNA from the body, the wounds were sealed with Vaseline immediately after the injection. Five pupae were injected with 8 μg siRNA each, whereas 25 pupae were injected with 4 μg or 2 μg siRNA each, separately. One set of siRNAs with random sequences were used as a negative control and injected alongside the experimental treatments. At 72 h after siRNA injection, 5 pupae were collected for qPCR and Western blot analysis. And the others were allowed growing under sterile conditions, hatched into moths for the observation of the wing changes in adult.

Results

The efficiency of the polyclonal antisera

The efficiency of prepared polyclonal mouse antiserum against BmHemolin or polyclonal rabbit antiserum against BmYippee was determined by Western blot analysis. As shown in Figure 1A, only a single band around 45 kDa was detected by antiserum against BmHemolin using total protein extraction from pupae as a loading sample (Lane 1), which is consistent with the predicted size (44.79 kDa) of silkworm hemolin. Meanwhile, a truncated peptide corresponding to hydrophilic region of BmHemolin expressed in *E. coli* BL21 was also detected as a major band around 21 kDa although there were some slight miscellaneous bands (Lane 2). It establishes that our produced polyclonal antiserum against BmHemolin could specifically recognize not only endogenous BmHemolin but also recombinant fragment of BmHemolin.

As for BmYippee rabbit polyclonal antiserum, a specific band of around 14 kDa was recognized (black arrow) using total protein extraction from pupae as a loading sample (Fig. 1B), which is consistent with the predicted size (13.72 kDa) of the silkworm Yippee protein. Our results indicate that both proteins are present in silkworm pupae, and both prepared antisera are suitable for future studies.

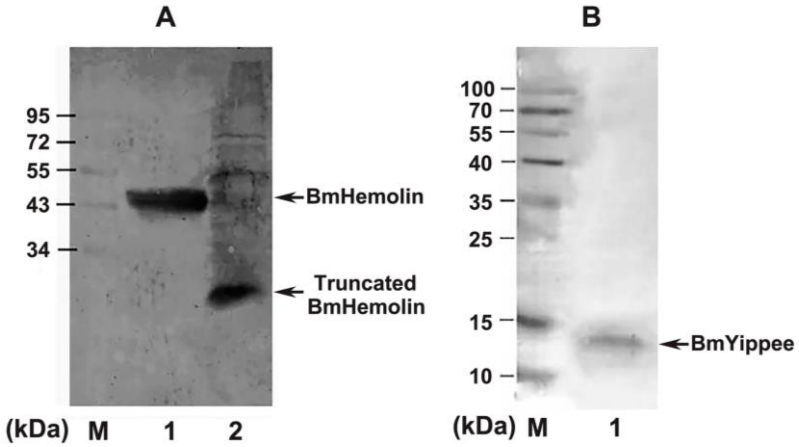

Fig. 1 Western blot analysis for the generation of polyclonal antisera against BmHemolin and BmYippee. A) Detection of BmHemolin by mouse polyclonal antisera against BmHemolin. Lane M, protein marker in kDa. Lane 1, total protein extraction from the silkworm pupae as a loading sample. Lane 2, a truncated peptide corresponding to hydrophilic region of BmHemolin expressed in *E. coli* BL21 as a loading sample. B) Detection of BmYippee by rabbit polyclonal antisera against BmYippee. Lane M, protein marker in kDa. Lane 1, total protein extraction from the silkworm pupae as a loading sample. The positions of detected proteins are indicated by the arrows.

Effects of xenobiotics on the expression of BmHemolin gene

Transcriptional changes in BmN cells

The BmN cells at about 80 % confluence were treated with xenobiotics by adding appropriate amount of inactivated *E. coli* (DH5α), peptidoglycan (PGN), and lipopolysaccharide (LPS) into the culture medium. After 24 h, the total RNA was extracted from cells, and qPCR was performed to determine the expression levels of Bm*Hemolin* gene. As shown in Figure 2, the transcription of Bm*Hemolin* gene in BmN cells did not appear significant changes, compared with the control group (PBS), which is completely different from the effects observed in the larval body.

Transcriptional changes in midgut tissues of larvae

On the third day of the fifth instar, the larvae were injected with inactivated *E. coli* (DH5α), BV virions of BmNPV, peptidoglycan (PGN), lipopolysaccharide (LPS), and PBS at indicated concentrations as described in Materials and Methods section. Total RNA was extracted from the midgut tissues of larvae at indicated time points and the results of qPCR are shown in Figure 3. Comparing with the control group (PBS), the transcriptional levels of Bm*Hemolin* in group DH5α were significantly increased by 21.97, 26.05, and 22.61 times at 9, 24, and 48 h post injection, respectively. For the group PGN and group LPS, the transcription of the Bm*Hemolin* gene was also up-regulated by the injection of peptidoglycan or lipopolysaccharide. However, the expression of Bm*Hemolin* gene did not appear to have any significant changes in the group BmNPV, with only a slight increase by 1.02, 1.11, and 1.07 times, at 9, 24 and 48 h post injection of virions, respectively, indicating that in silkworm, the BmNPV cannot induce Bm*Hemolin* transcription.

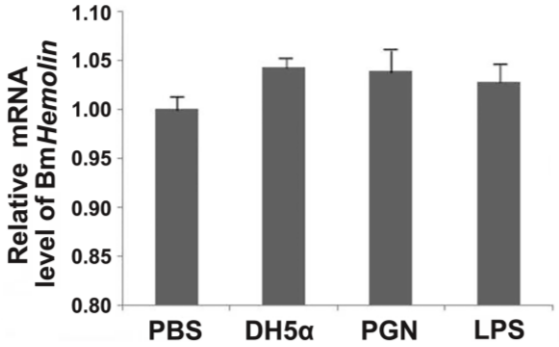

Fig. 2 Transcriptional changes of Bm*Hemolin* gene from stimulants challenged BmN cells. A qPCR was performed at 24 h after adding exogenous substances in triplicate for each treatment. Expression values were normalized to those of *α-tubulin* using the Livak ($2^{-\Delta\Delta CT}$) method and the data were provided as the mean fold changes (means ± SD, n = 3) relative to the control group (PBS). The vertical axis indicates a relative mRNA level of Bm*Hemolin* and the horizontal axis shows the addition of PBS (PBS), inactivated *E. coli* (DH5α), peptidoglycan (PGN), and lipopolysaccharide (LPS), respectively, into the cultured medium.

Effects of xenobiotics on the changes of protein level of BmHemolin in the silkworm midgut

Following the same procedure as described above, on the third day of the fifth instar, the larvae were injected with inactive *E. coli*, BmNPV, PGN, LPS and PBS. At 9, 24, and 48 h post injection, total proteins were extracted from the midgut tissues. Western blot analysis was performed using polyclonal antiserum anti-BmHemolin as the primary

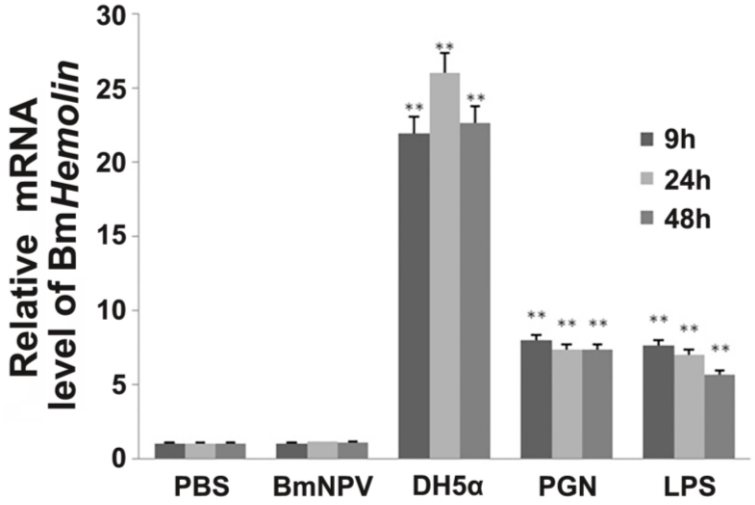

Fig. 3 qPCR analysis for the transcriptional changes of Bm*Hemolin* from stimulants challenged midgut at larval stage. A qPCR was performed at 9, 24, and 48 h, respectively, from stimulants challenged midgut tissues of silkworm at the fifth instar larvae. Expression values were normalized to those of *α-tubulin* using the Livak ($2^{-\Delta\Delta CT}$) method the data were provided as the mean fold changes (means ± SD, n = 10) relative to the control group (PBS). The injected stimulants shown on the horizontal axis are PBS as control, BV virions (BmNPV), inactivated *E.coli* (DH5α), peptidoglycan (PGN), and lipopolysaccharide (LPS), respectively.

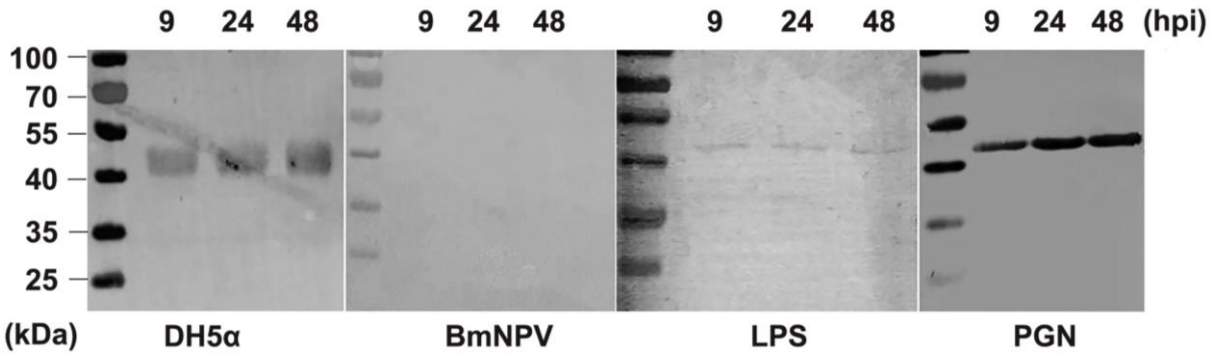

Fig. 4 Western blot analysis for the expression of BmHemolin protein from stimulants challenged midgut at larval stage. Parallelly, a Western blot analysis was performed at 9, 24, and 48 h from stimulants challenged midgut tissues of silkworm at the fifth instar larvae, respectively, as described in "Materials and Methods" section. Numbers on the left indicate a protein marker in kDa. The injected stimulants at the bottom are inactive *E. coli* (DH5α), *Bombyx mori* Nuclear Polyhedrosis Virus (BmNPV), lipopolysaccharide (LPS), and peptidoglycan (PGN), respectively

antibody to detect BmHemolin protein changes in the silkworm midgut tissues. The results are shown in Figure 4. During 48 h post injection of inactive *E. coli*, BmHemolin protein levels were significantly increased at different time points (9, 24, and 48 h) (Fig. 4A). However, there was no any BmHemolin protein to be observed by the injection with *B. mori* NPV (Fig. 4B). Injection of LPS or PGN resulted in a moderate increase in BmHemolin expression (Figs 4C, D). These results are consistent with the observations on the changes of mRNA levels after xenobiotics injections in which the stimulating effect of inactivated *E. coli* was much higher than that by LPS or PGN injection.

Interaction between BmHemolin and BmYippee in the wing disc tissue

The interaction of BmHemolin and BmYippee in *B. mori* wing disc tissue was verified by Co-IP assays. Immunoprecipitation with anti-BmHemolin or anti-BmYippee antisera was performed using total tissue lysates from wing disc, respectively, as shown in Figure 5. The carefully washed immunoprecipitates were then subjected to SDS-PAGE electrophoresis. As indicated by black arrows on Coomassie blue-stained gel, a separated band from immunoprecipitates with anti-BmHemolin antisera corresponding to the position of BmYippee protein (Fig. 5A, Lane 1) or the one from immunoprecipitates with anti-BmYippee antisera corresponding to BmHemolin (Fig. 5B, Lane 1) was excised using a sterile scalpel blade and followed by MALDI-TOF mass-spectrometric analysis (Shanghai Applied Protein Technology Co. ltd, Shanghai, China). Two bands were identified as BmHemolin and BmYippee (data not show), implying that there is a direct interaction between two proteins in the wing tissue.

To further verify the above results, the Western blot analysis was performed. The immunoprecipitates from total wing disc tissue lysates with anti-BmHemolin antisera or with anti-BmYippee antisera were run on a 12 % SDS-PAGE gel, separately. The separated proteins were then transferred onto a nitrocellulose membrane. The anti-BmYippee antiserum was used as a primary antibody to detect BmYippee protein from co-immunoprecipitates with anti-BmHemolin antisera. Parallelly, an anti-BmHemolin antiserum was used to detect BmHemolin protein from co-immunoprecipitates with anti-BmYippee antisera. Correspondingly, a single band of BmYippee or BmHemolin protein was detected as shown in Figures 5C and D. Therefore, there is an interaction between hemolin and yippee proteins in the wing disc of silkworm.

RNA interference of Hemolin *in the wing disc at pupal stage*

*The expression of Bm*Hemolin *gene after RNA interference*

The siRNA solution was injected into each pupa at the position of wing disc. After 72 h, the total RNA was extracted and the changes of Bm*Hemolin* transcription were determined by qPCR. In an initial round of experiments, the injection of a high dose (8 μg/pupa) led to death of almost all the pupae. Therefore, an injection with a low dose (2 μg/pupa) or mid dose (4 μg/pupa) was applied in subsequent experiments. The qPCR results for Bm*Hemolin* gene transcription in the pupal stage are shown in Figure 6A. Comparing with the negative control group (Lane N), strangely, there were no significant effects on Bm*Hemolin* gene transcription by the injection of 2 μg or 4 μg per pupa with three different pairs of specific BmHemolin-siRNA (Lanes 1 - 3).

As shown in Figure 6B, the protein levels of BmHemolin after injection of siRNAs in the pupa wing disc were also determined by Western blot analysis. Comparing with the control group (Lane 1), the protein levels were slightly decreased by a 2-μg/pupa injection with either pair 1 (RNAiBmHem-1) or pair 2 (RNAiBmHem-2) of specific BmHemolin-siRNA (Lane 2 and 3). Interestingly, the protein levels were dramatically decreased to almost

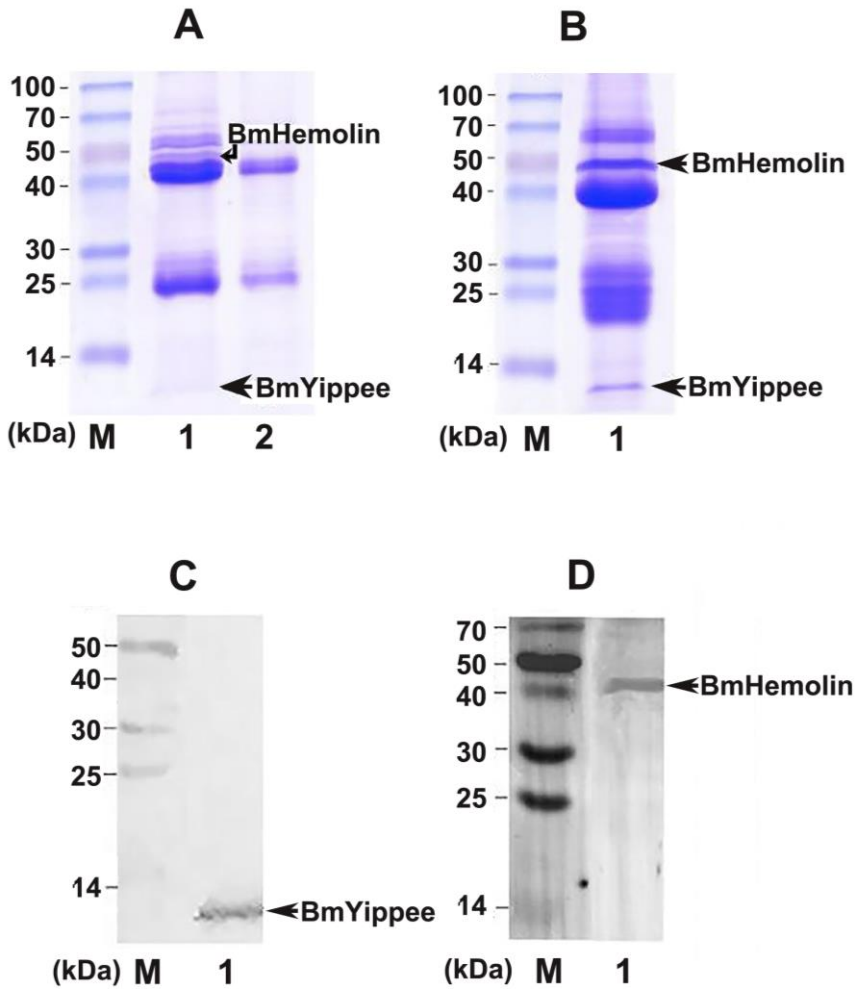

Fig. 5 Co-IP assays for the interaction of BmHemolin and BmYippee in the wing disc tissue. A and B: Coomassie Blue stained SDS-PAGE for Co-IP assays. A) Co-IP assays were performed using total tissue lysates from wing disc with mouse anti-BmHemolin polyclonal antisera (Lane 1) in which a mouse monoclonal antibody anti-Vta1 was used as the control (Lane 2). B) A revised Co-IP was performed using total tissue lysates from wing disc with rabbit anti-BmYippee polyclonal antisera (Lane 1). C and D) Western blot analysis for Co-IP. The immunoprecipitates with mouse anti-BmHemolin antisera or with rabbit anti-BmYippee antisera were then followed by immunodetection with rabbit anti-BmYippee antisera (C) or with mouse anti-BmHemolin antisera (D). Lane M indicates protein marker in kDa. The positions of BmHemolin and BmYippee proteins are marked by arrows.

undetectable levels by a 4-μg/pupa injection of either pair 1 or pair 2 siRNA (Lane 6 and 7). For pair 3 siRNA, both 2-μg and 4-μg injection per pupa did not lead to significant decreases in protein levels (Lane 4, 8). Therefore, the injection of 4-μg siRNAs of pair 1 or pair 2 to each pupa was applied to observe the effects of decreased BmHemolin on the development of adult wings.

Phenotypic changes in adult wings after RNA interference

Apart from those siRNA-injected pupae that were collected at 72 h post injection for qPCR and Western blot analysis, the rest of them continuously grew and eventually hatched into moths, and the phenotypic changes in adult wing were observed. By a low dose of injection (2 μg/pupa) with three different pairs of siRNAs, no significant changes of adult wing were observed. However, by an injection of 4 μg/pupa dose with pair 1 or pair 2 siRNA, the developed wings were not normal while there were no any phenotypic changes in other body parts. Most of them tended to be in a short, shrinked, and unopened state, in which the injection of pair 2 was found more effective than that by the injection of pair 1 (Fig. 7). Among 20 hatched moths by the injection (4 μg/pupa) of pair 2 siRNA, 8 moths exhibited abnormal wing development. From a repeated experiment using 10 pupae by pair 2 siRNA injection (4 μg/pupa), 5 hatched moths were presented as abnormal, strongly suggesting that BmHemolin plays an important role in adult wing development. As for 4-μg/pupa dose of injection with pair 3, the adult wings did not change significantly compared with the negative control group.

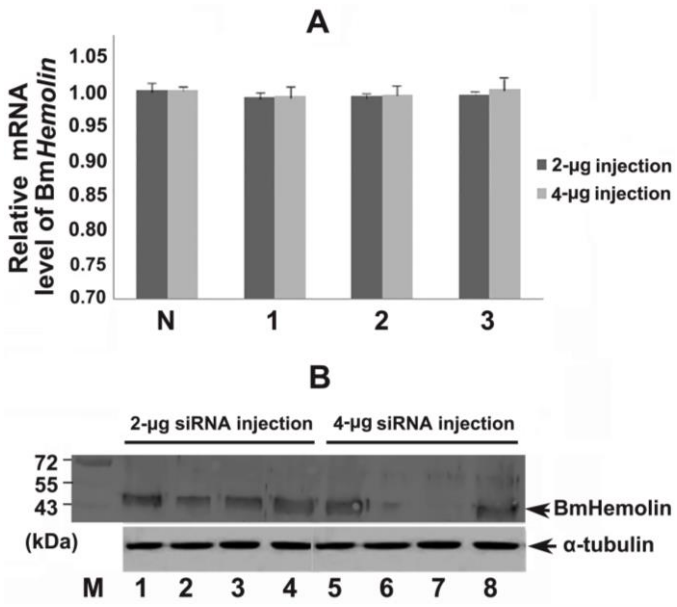

Fig. 6 RNA interference of the Bm*Hemolin* gene. A) The relative expression of Bm*Hemolin* mRNA at 72 h post injection of siRNA. A siRNA (2 or 4 μg) specific to BmHemolin was injected into each pupa at the position of wing disc. The expression levels were assessed using siRNA random sequences for normalization. Lane N, 1, 2, and 3 indicate that the injected siRNA was random sequences, pair 1, pair 2, and pair 3, respectively, as described in Table 1. B) Western blot analysis of the BmHemolin protein levels in response to RNAi by an injection of specific BmHemolin-siRNA at 72 h. M, protein marker in kDa. Lane 1 - 4, 2-μg injections with random sequences, pair 1, pair 2, and pair 3, respectively. Lane 5 - 8: 4-μg injections with the same siRNA as that of 2-μg injections. The α-tubulin protein was used as a loading control.

Discussion

In insects, innate immunity and development often share the same molecules, for example, sapecin and lectin protein in *Sarcophaga peregrina*. They are up-regulated upon pathogen stimulation, mainly expressed in the fat body which is functional equivalent to mammalian liver and/or kidney and in some hemocyte species. Meanwhile, they are also found to be expressed at specific developmental stages (Natori, 2010). These proteins seem to play two independent roles. Hemolin is such a kind of dual-function molecule. However, the addition of inactivated *E. coli*, bacterial cell wall components, LPS and PGN, into cultured medium did not stimulate the expression of *hemolin* gene in BmN cells in this work (Fig. 2). Therefore, the pathogen cannot directly stimulate *hemolin* expression in cultured BmN cells.

Normally, hemolin works as a pattern recognition protein for identifying the infection source via cell signal transduction, and is engaged in phagocytosis to eliminate xenobiotics. It is proposed that hemolin is stimulated by exogenous pathogen in fat body and then released into the hemolymph where hemolin protein is capable of identifying the bacterial cell wall components, lipopolysaccharide and peptidoglycan, and gathering pathogenic microorganisms to eliminate pathogens (Yu and Kanost, 2002). Truly, our studies confirm that the expression of *hemolin* gene could be up-regulated by the injection of inactivated bacteria or bacterial cell wall components (LPS and PGN) into the midgut of fifth-instar silkworm larvae (Fig. 3). As for LPS and PGN injection, the up-regulation of *hemolin* gene expression was higher in the PGN injection group than that in LPS injection group. A possible explanation is the high molecular weight of PGN. Alternatively, an amino acid moiety in side chain, a part of the short peptide in PGN, serves as an easily recognizable pattern for the insect immune system, and therefore, results in a strong induction of *hemolin* gene expression. Also, hemolin plays an antiviral role in some species, but in some other species, it has no effect on viral invasion. For instance, it is involved in antiviral resistance in *Bombycoidea*, but not in *Noctuoidea* (Terenius *et al*., 2009). Our experiment indicates that the *B. mori* nuclear polyhedrosis virus did not stimulate the up-regulation of *hemolin* gene expression in the midgut of fifth-instar silkworm larvae (Fig. 3).

The first identification of *Drosophila* yippee as an interaction protein of *H. cecropia* hemolin is from the proteins encoded in a *Drosophila* imaginal disc cDNA library (Roxstrom-Lindquist and Faye, 2001). Therefore, we applied Co-IP assay, using our prepared two polyclonal antisera with a high sensitivity and specificity in detection of endogenous BmYippee and BmHemolin (Fig. 1), to verify their interaction in silkworm wing discs. Our study demonstrates that there is a direct interaction between two proteins in the wing disc of silkworm (Fig. 5).

Fig. 7 Morphological changes in adult wings after RNA interference. The pupae were injected by 4-μg pair 1 or pair 2 of siRNA and eventually hatched into moths. Panel 1, no siRNA injection as the control (a normal silkworm wing development state). Panel 2, 4 μg of siRNA injection with pair 1. Panel 3, 4 μg of siRNA injection with pair 2.

The effects of hemolin on the wing disc development were further investigated in silkworm pupae by preliminary siRNA interference. As expected, hemolin protein levels were dramatically decreased by an injection of 4 μg/pupa dose with pair 1 or pair 2 siRNA in pupal stage (Fig. 6, Lanes 6, 7), which leads to subsequent adult wings hypoplasia in most hatched moths (Fig. 7, panels 2, 3). It strongly suggests that Hemolin plays a crucial role in adult wing development of silkworm. The puzzling observation was that the fluorescence qPCR results showed no significant reduction in Bm*Hemolin* gene transcription by siRNA interference in pupal stage although a slight decrease was observed by the injection with pair1 or pair 2 siRNA (Fig. 6A). One possible reason is that our samples were taken from the parts of pupae which are not separated from the wing tissue. Therefore, further experiments are required by the use of a sample specially isolated from wing tissues to confirm it.

Both the protein and gene structure of hemolin are similar to cell adhesion proteins (Kanost *et al*., 1994; Bettencourt *et al*., 1997). In fact, hemolin can function as a cell adhesion molecule in the immune system. But further experiments are needed to elucidate the role of hemolin in the development of insects to see whether the *hemolin* gene is associated with tissue reconstruction and how to play a functional role in insect development. Thus, our works will be significant in studies on the functional role of hemolin in silkworm immunological and developmental processes in the future, enriching our knowledge of the immune regulation and mechanisms of insect metamorphosis.

Conclusions

Hemolin can work as a pattern recognition protein for identifying the infection source via cell signal transduction, and is engaged in phagocytosis to eliminate xenobiotics; hemolin plays an important role in development of wing disc of silkworm.

Acknowledgements

This work was supported by National Natural Science Foundation of China (31101673, 31372259); Natural Science and Youth support Foundation for Universities of Jiangsu (1281330023, 5621330001); Natural Science foundation of Jiangsu province (BK20140539) and funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). We wish to thank Dr. Yajing Z for reorganizing and revising the manuscript.

References

Bettencourt R, Lanz-Mendoza H, Lindquist KR, Faye I. Cell adhesion properties of hemolin, an insect immune protein in the Ig superfamily. Eur. J. Biochem. 250: 630-637, 1997.

Bettencourt R, Terenius O, Faye I. Hemolin gene silencing by ds-RNA injected into Cecropia pupae is lethal to next generation embryos. Insect Mol. Biol. 11: 267-271, 2002.

Daffre S, Faye I. Lipopolysaccharide interaction with hemolin, an insect member of the Ig-superfamily. FEBS Lett. 408: 127-130, 1997.

Faye I, Pye A, Rasmuson T, Boman HG, Boman IA. Insect immunity. 11. Simultaneous induction of antibacterial activity and selection synthesis of some hemolymph proteins in diapausing pupae of Hyalophora cecropia and *Samia cynthia*. Infect Immun. 12: 1426-1438, 1975.

Golemis E. Protein-protein interactions: A molecular cloning manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. ix, 682, 2002.

Hosono K, Noda S, Shimizu A, Nakanishi N, Ohtsubo M, Shimizu N, *et al.* YPEL5 protein of the YPEL gene family is involved in the cell cycle progression by interacting with two distinct proteins RanBPM and RanBP10. Genomics 96: 102-111, 2010.

Hosono K, Sasaki T, Minoshima S, Shimizu N. Identification and characterization of a novel gene family YPEL in a wide spectrum of eukaryotic species. Gene 340: 31-43, 2004.

Kanost MR, Zepp MK, Ladendorff NE, Andersson LA. Isolation and characterization of a hemocyte aggregation inhibitor from hemolymph of *Manduca sexta* larvae. Arch. Insect Biochem. Physiol. 27: 123-136, 1994.

Kirkpatrick BA, Washburn JO, Volkman LE. AcMNPV pathogenesis and developmental resistance in fifth instar *Heliothis virescens*. J. Invertebr. Pathol. 72: 63-72, 1998.

Liang P, Wan Y, Yan Y, Wang Y, Luo N, Deng Y, *et al*. MVP interacts with YPEL4 and inhibits PEL4-mediated activities of the ERK signal pathway. Biochim. Cell Biol. 88: 445-450, 2010.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.

Natori S. Molecules participating in insect immunity of *Sarcophaga peregrina*. Proc. Jpn. Acad. Ser. B Physiol. Biol. Sci. 86: 927-938, 2010.

Roxstrom-Lindquist K, Assefaw-Redda Y, Rosinska K, Faye I. 20-Hydroxyecdysone indirectly regulates Hemolin gene expression in *Hyalophora cecropia*. Insect Mol. Biol. 14: 645-652, 2005.

Roxstrom-Lindquist K, Faye I. The *Drosophila* gene Yippee reveals a novel family of putative zinc binding proteins highly conserved among eukaryotes. Insect Mol. Biol. 10: 77-86, 2001.

Schurmann G, Haspel J, Grumet M, Erickson HP. Cell adhesion molecule L1 in folded (horseshoe) and extended conformations. Mol. Biol. Cell 12: 1765-1773, 2001.

Sun SC, Boman HG, Faye I, Schmidt O. Hemolin: an insect-immune protein belonging to the immunoglobulin superfamily. Science 250: 1729-1732, 1990.

Terenius O, Bettencourt R, Lee SY, Li W, Soderhall K, Faye I. RNA interference of Hemolin causes depletion of phenoloxidase activity in *Hyalophora cecropia*. Dev. Comp. Immunol. 31: 571-575, 2007.

Terenius O. Hemolin-A lepidopteran anti-viral defense factor? Dev. Comp. Immunol. 32: 311-316, 2008.

Terenius O, Popham HJR, Shelby KS. Bacterial, but not baculoviral infections stimulate Hemolin expression in noctuid moths. Dev. Comp. Immunol. 33: 1176-1185, 2009.

Xin W, Jie M, Tejun S. Expression analysis and subcellular localization of BmYippee from silkworm,*Bombyx mori*. Master Thesis. Zhejiang Sci-Tech University, 2009.

Yu XQ, Kanost MR. Binding of hemolin to bacterial lipopolysaccharide and lipoteichoic acid. An immunoglobulin superfamily member from insects as a pattern-recognition receptor. Eur. J. Biochem. 269: 1827-1834, 2002.

Zuo H, Li H, Wei E, Su Z, Zheng J, Li C, *et al.* Identification and functional analysis of a Hemolin like protein from *Litopenaeus vannamei*. Fish Shellfish Immunol. 43: 51-59, 2015.