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Silkworm ferritin 1 heavy chain homolog is involved in defense against bacterial infection through regulation of haemolymph iron homeostasis

Sohail Ahmed Otho [a, 1], Kangkang Chen [a, 1], Yongdong Zhang [a], Peng Wang [a], Zhiqiang Lu [a, b, *]

[a] Department of Entomology, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi 712100, China
[b] Key Laboratory of Plant Protection Resources and Pest Management, Ministry of Education, Northwest A&F University, Yangling, Shaanxi 712100, China

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ABSTRACT

Iron functions as a nutrient and a potential toxin in all organisms. It plays a key role in the interaction between microbes and their hosts as well. Microbial infection disrupts iron homeostasis in the host; meanwhile the host endeavors to keep the homeostasis through iron transport and storage. Transferrins and ferritins are the major iron-binding proteins that affect iron distribution in insects. In this study, we investigated a possible involvement of *Bombyx mori* ferritin 1 (BmFer1) heavy chain homolog in the defense against bacterial infection in the silkworm larvae. The *BmFer1* mRNA abundance was up-regulated in hemocytes, but not in fat body, after *Pseudomonas aeruginosa* or *Staphylococcus aureus* infection. The infection resulted in elevated iron levels in the hemolymph. Injection of recombinant BmFer1 protein into hemocoel reduced the plasma iron level after infection, limited the bacterial growth in the hemolymph, and resulted in a lower mortality caused by infection. Our study indicated that *B. mori* ferritin-1 may restrict iron access of the invading bacteria to block their growth as a defense strategy.

1. Introduction

Iron is an essential nutrient for almost all living organisms, playing essential roles as cofactors of many enzymes that are responsible for basic metabolic pathways and cellular processes. Iron can be a potential toxin through the Fenton reaction. Free iron catalyzes the formation of oxygen free radicals that cause protein denaturation, DNA breakage, and lipid peroxidation. Iron levels also modulate host–pathogen interactions. Depletion of free iron impaired the production of infectious progeny of *Chlamydia pneumoniae* (Al-Younes et al., 2001). Infection causes iron level changes in the hosts. For instance, fungal infection by *Beauveria bassiana* resulted in an elevated level of iron in the hemolymph of a larval beetle *Protaetia brevitarsis* (Kim et al., 2008). Similarly, iron levels of *Heliothis virescens* larval hemolymph increased following baculovirus infection (Popham et al., 2012). The change of iron levels in these hosts was a result of the interaction between hosts and pathogens. The pathogens despoil iron from the host tissues to support their growth in the hosts; the hosts maintain iron at a relatively stable level and restrict the pathogens' access to iron (reviewed by Schaible and Kaufmann, 2004; Ong et al., 2006; Ganz, 2009; Armitage and Drakesmith, 2014).

Two families of proteins, transferrins and ferritins, transport and store iron in insects (Nichol et al., 2002; Pham and Winzerling, 2010; Tang and Zhou, 2013a; Mandilaras et al., 2013). In addition, insect transferrins act as antioxidants (Kim et al., 2008) and antimicrobial proteins (Yun et al., 2009). Like vertebrate ferritins, insect ferritins are composed of two types of subunits, heavy chain homologs (HCHs) and light chain homologs (LCHs) (Hamburger et al., 2005). The HCHs are characterized with the presence of a ferroxidase center responsible for the oxidation of Fe^{2+} to Fe^{3+} (Missirlis et al., 2007). Most insect ferritins are secreted proteins and abundant in the plasma, suggestive of regulatory roles in iron hemeostasis (Nichol et al., 2002; Missirlis et al., 2007; Tang and Zhou,

Abbreviations: CFU, colony formation unit; URE, iron-responsive element; IRPs, iron regulatory proteins; IPTG, isopropy-β-D-thiogalactoside; LB, Luria-Bertani; LPS, lipopolysaccharide; qRT-PCR, quantitative real-time polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

* Corresponding author. Department of Entomology, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi 712100 China.
E-mail address: zhiqiang.lu@nwsuaf.edu.cn (Z. Lu).

[1] These authors made equal contribution to this work.

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2013a). The *Drosophila melanogaster* secretory ferritin is indispensable for dietary iron absorption and cellular iron detoxification (Tang and Zhou, 2013b). The bumblebee (*Bombus ignites*) transferrin and ferritin HCH transcription was induced by iron overload (Wang et al., 2009). Iron overload also induced the oriental fruit fly (*Bactrocera dorsalis*) ferritin 1 HCH and ferritin 2 LCH (Jiang et al., 2014).

In the proteomic studies of *Drosophila* hemolymph, it was found that transferrin 1 level increased after *B. bassiana* infection; ferritin 1 HCH decreased after the fungal infection but increased after infection/challenge by *Micrococcus luteus*, *Escherichia coli*, *Saccharomyces cerevisiae* (Levy et al., 2004; Vierstraete et al., 2004a), and lipopolysaccharide (LPS) (Vierstraete et al., 2004b). In the flesh fly (*Sarcophaga bullata*), a ferritin-like protein decreased in larval fat body after *E. coli* infection (Masova et al., 2010). A transcriptional analysis revealed that *Aedes aegypti* ferritin HCH and LCH expression levels were slightly upregulated in the midgut 24 h after a challenge with heat-killed bacteria (Geiser et al., 2013). Upon infection by the microsporidian parasite *Edhazardia aedis* or *Vavraia culicis*, the mosquito showed a decreased amount of ferritin HCH (Duncan et al., 2012). In oriental river prawn (*Macrobrachium nipponense*) and rock bream (*Oplegnathus fasciatus*), ferritin HCH transcriptional levels were upregulated by immune challenges (Sun et al., 2014; Elvitigala et al., 2014). Together, these studies suggest a role of ferritins in the regulation of iron homeostasis during pathogen-host interaction.

The silkworm *Bombyx mori* transferrin mRNA expression is upregulated by LPS, peptidoglycan, laminarin, and slightly by virus. The recombinant silkworm transferrin exhibits antibacterial activity (Yun et al., 2009). A study on silkworm ferritin 2 LCH indicates that it is unlikely involved in defense against bacterial and fungal infection (Hong et al., 2014). In this study, we show that silkworm ferritin 1 HCH (*BmFer1*) transcriptional expression is induced in the hemocytes after bacterial infection. The recombinant BmFer1 is able to chelate iron in hemolymph, hinder bacterial growth and proliferation, and consequently mitigate the larvae mortality caused by infection.

2. Materials and methods

2.1. Insect rearing, bacterial challenge, and tissue collection

The silkworm *B. mori* (*Nistari* strain) was reared on mulberry leaves at 27 °C in 70% relative humidity and a photoperiod of 12 h light: 12 h dark. The overnight cultured *Pseudomonas aeruginosa* and *Staphylococcus aureus* cells were harvested by centrifugation at 8000*g* for 15 min. The bacteria were washed three times and then suspended with sterilized 0.85% NaCl. Day 3, 5th instar larvae were used for bacterial infection. The infection groups were injected with 1×10^7 cells of *P. aeruginosa* or *S. aureus*in in 50 μl sterilized 0.85% NaCl, and the control group was injected with 50 μl sterilized 0.85% NaCl saline. There were 5 larvae in each group. At 6, 12, 24 and 48 h after the larvae were infected, the silkworms in each group were dissected to collect fat body and hemocytes. The collected tissues were stored in Trizol regent at −80 °C. Each treatment was repeated three times.

2.2. RNA extraction, cDNA preparation and qRT-PCR analysis

Total RNA was extracted from the collected tissues according to the manufacturer's instructions. The isolated RNA was purified using the Direct-zol RNA MiniPrep Kit (Zymo) and used as template for first-strand cDNA synthesis by M-MLV Reverse Transcriptase according to the manufacturer's instructions (Promega). The cDNA was then used as the template for qRT-PCR analysis of *BmFer1* expression. The forward (5′-CGATGCTGCGACTGAAGA-3′) and reverse (5′-GACCTCCCGGATGCTGTT-3′) primers were for *BmFer1*. The forward (5′- AATCAACGTGAGCGTGAAGTG-3′) and reverse (5′-TCCCTTACGACCAAAACGTC -3′) primers were for *BmIF4A* (DQ443290.1), the internal control (Wu et al., 2010). qRT-PCR was performed on a Rotor Q (Qiagen, Germany) under the following conditions: 95 °C for 10 min, 40 cycles (95 °C for 20 s, 59 °C for 35 s), and a transition from 65 to 95 °C for melting curve determination. The results were analyzed by the relative quantitative method ($2^{-\Delta\Delta Ct}$) (Schmittgen and Livak, 2008) and plotted using Prism 5.0 (GraphPad Software, Inc). Statistical differences were analyzed by student's *t*-test.

2.3. Cloning, expression and purification of recombinant BmFer1

The coding region of mature *BmFer1* (^{21}TQCYV————LGLNV^{209}, Fig. 1) was amplified using two specific primers (forward: 5′-AGCATATGACACAGTGTTACGTCAGCC-3′ with an *Nde*I site and reverse: 5′-GAAGCTTATACGTTCAATCCGAGGAG-3′ with a *Hin*dIII site). The amplified fragment was inserted into pMD 19-T vector (Takara, Dalian, China) and the sequence was confirmed by sequencing. After digestion with *Nde*I and *Hin*dIII enzymes, the fragment was subcloned into the same sites in the expression vector pET-28a (Novagen) to transform *E. coli* BL21 (DE3). The transformants were cultured in 1 L of Luria Bertani (LB) medium containing 50 μg/ml kanamycin at 37 °C with shaking at 220 rpm, and then induced by isopropy-β-D-thiogalactoside (IPTG) at a final concentration of 0.5 mM for 24 h at 25 °C with shaking at 150 rpm. The bacteria were harvested and lysed as described before (Chen et al., 2014). The inclusion bodies were collected after centrifugation at 14,500*g* for 15 min at 4 °C, and then dissolved in 10 ml phosphate buffered saline (pH 8.5) containing 8 M urea. After centrifugation at 14,500*g* for 15 min, BmFer1 in the supernatant was purified on a Ni^{2+}-NTA agarose column (Qiagen) eluted with 0.05 M, 0.15 M and 0.3 M imidazole in PBS containing 8 M urea. The purified BmFer1 was dialyzed in PBS (pH 8.5) containing 6.0, 3.0, 2.0, 0.5 and 0 M urea at 4 °C to refold the denatured BmFer1. To monitor the purification, proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Brilliant Blue R250 and immunoblotting using anti-hexahistidine (1:10,000 dilution, Santa Cruz) as the primary antibody and goat-anti-rabbit IgG (1:10,000 dilution, Cwbio) as the secondary antibody.

2.4. Iron chelating assay

The assay for iron chelating ability of recombinant BmFer1 was modified from De Zoysa and Lee (2007) and Ren et al. (2014). CO_2 was introduced into the reaction mixture to protect $FeCl_2$ from oxidation. Bovine serum albumin (BSA) and ferritin from horse spleen (Sigma-F4503) were used as controls. BmFer1, horse ferritin and BSA were diluted to 18.75, 75, 150 μg/ml. The diluted proteins (250 μl) and 10 μl of 2 mM $FeCl_2$ were incubated at 22 °C for 10 min. Then 20 μl of 5 mM Ferrozine (Sigma, USA) was added to the mixture. After incubation for 15 min, absorbance at the 550 nm was measured.

2.5. Plasma total iron concentration assay

To determine total iron concentration in plasma after bacterial infection, we collected hemolymph from silkworm larvae at 2, 4, 8 and 24 h after injection of bacteria. The hemolymph samples were centrifuged at 500*g* for 10 min at 4 °C to remove hemocytes. The supernatants were used for iron concentration assays with QuantiChrom™ Iron Assay Kit (BioAssay Systems). The kit contains a