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Isolation of ferritin and its interaction with BmNPV in the silkworm, *Bombyx mori*

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A B S T R A C T

Ferritin is a ubiquitous iron storage protein that plays an important role in host defence against pathogen infections. In the present study, native ferritin was isolated from the hemolymph of *Bombyx mori* using native-polyacrylamide gel electrophoresis (native-PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The results revealed that ferritin consisted of two subunits, designated as BmFerHCH and BmFerLCH. Previously integrated previous transcriptome and iTRAQ data showed that the two subunits were down-regulated in resistant silkworm strain BC9 and there was no obvious change in the expression levels of the subunits in susceptible silkworm strain P50 after BmNPV infection. Virus overlay assays revealed that *B. mori* ferritin as the form of heteropolymer had an interaction with *B. mori* nucleopolyhedrovirus (BmNPV), but it can't interact with BmNPV after depolymerisation. What's more, reverse transcription quantitative PCR (RT-qPCR) analysis suggested that *BmFerHCH* and *BmFerLCH* could be induced by bacteria, virus and iron. This is the first study to extract *B. mori* ferritin successfully and confirms their roles in the process of BmNPV infection. All these results will lay a foundation for further research the function of *B. mori* ferritin.

A R T I C L E I N F O

Keywords:
Ferritin
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B. mori nucleopolyhedrovirus
Interaction
Virus overlay assay

1. Introduction

The silkworm *Bombyx mori* (*B. mori*) is a holometabolous lepidopteran insect that has been domesticated for about 5700 years from the wild progenitor *Bombyx mandarina* (Xia et al., 2004). It is used as a model organism in fundamental research and applied biotechnology (Kato et al., 2010). *B. mori* nucleopolyhedrovirus (BmNPV) is a serious viral pathogen that specifically infects the domestic silkworm, causing severe economic loss in sericulture around the world (Hu et al., 2016). In the process of BmNPV infection, two different virion phenotypes are produced: budded virus (BV) and occlusion-derived virus (ODV). ODV causes an infection between individuals, while BV causes the system to spread all over the host (Dong et al., 2016). In recent years, many proteins involved in BmNPV infection have been identified. *B. mori* receptor expression enhancing protein a (BmREEPa) can interact with glycoprotein 64 to participate in BV invasion (Dong et al., 2017). Membrane lipid raft-related protein methyl-β-cyclodextrin (MβCD) also plays an important role in the process of BmNPV entry into BmN cells (Hu et al., 2017). It has been reported that human ferritin concentrations increases with human immunodeficiency virus (HIV) progression (Savarino et al., 1999). Iron also enhances hepatitis C virus (HCV) infection in the liver (Kakizaki et al., 2000). These results indicate that iron metabolism is essential for virus-host interaction.

Iron is an essential nutrient required in all living organisms, and it plays an important role in metabolism and homeostasis. It is also used as a co-factor in various biological processes, such as respiration, photosynthesis, and hydroxylation reactions (Stiles et al., 2009; Semenza, 2009). However, iron is highly toxic when it is present in excess. The production of free intracellular Fe^{2+} can catalyse the formation of toxic reactive oxygen species (ROS) via a Fenton reaction

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(Andrews, 2000). The hydroxyl radical is an extremely powerful oxidising agent that is capable of causing extensive cell damage (Corbett, 1995). Therefore, the tight regulation of iron metabolism, which is necessary to maintain balance in host cells, is performed by the iron-binding proteins (IBPs) such as ferritin and transferrin (Otho et al., 2016).

In insects, ferritin is an iron-binding protein, and it is a highly-conserved multifunctional protein (Arosio et al., 2009). It plays a critical role in cellular and organismal iron homeostasis by capturing the intracellular iron pool and protecting the cell from damage by excess iron (Kakhlon et al., 2001; Konijn et al., 1999). Ferritin is found in almost all major clades of organisms, including bacteria, fungi, plants, and animals (Shi et al., 2008). The typical ferritin protein is composed of 24 subunits, which are arranged in 4,3,2 symmetry to form a spherical and hollow complex with an approximately 8 nm diameter cavity that is capable of storing up to 4500 iron atoms (Hamburger et al., 2005; Theil, 1987). Insect ferritin polymers are composed of two types of subunits: (1) a heavy-chain homolog (HCH) that contains a ferroxidase centre and that catalyses the oxidation of ferrous iron, and (2) a light chain homolog (LCH) with amino acid residues that can facilitate iron nucleation and protein stability (Santambrogio et al., 1993; Orino et al., 2001; Theil, 2011). Insect ferritins also have multiple functions, such as storing and releasing iron, playing a role in embryonic and post-embryonic development, acting as a cytotoxic protector against oxidative stress and acting as a potential antimicrobial effector (Anderson and Frazer, 2005; Pham and Winzerling, 2010; Gutierrez et al., 2013; Colinet et al., 2013). *Drosophila* hemolymph proteome analyses indicate that ferritin expression is up-regulated after infection by bacteria and lipopolysaccharide (LPS), and down-regulated after fungal infection (Pham and Winzerling, 2010; Gutierrez et al., 2013). Injection of recombinant ferritin 1 heavy chain homolog (BmFer1) into silkworms infected with *Pseudomans aeruginosa* (*Pa*) and *Staphylococcus aureus* (*Sa*) resulted in markedly improved lower bacterial loads, indicating an functional role of BmFer1 in *B. mori* immunity (Otho et al., 2016). However, the role of ferritin after BmNPV infection and the interaction between BmNPV and ferritin have never been reported.

The major target of present study was to isolate ferritin from the hemolymph of fifth-instar larvae of *B. mori*, and to analyse the potential functions of ferritin in the immune response to various pathogens. Our results showed that the molecular weight (MW) of *B. mori* native ferritin was about 670 kDa, and it consisted of two subunits (BmFerHCH and BmFerLCH). The expression profiles of *BmFerHCH* and *BmFerLCH* were examined in different tissues and at different developmental stages, as well as after iron overload and challenges to BmNPV, *P. aeruginosa* and *S. aureus* challenge. A virus overlay assay was conducted to screen for a ferritin heteropolymer that could combine directly with the BmNPV. The results suggested the involvement of *B. mori* ferritin in the innate immune defence against invading pathogens.

2. Materials and method

2.1. Silkworm rearing and BmNPV preparation

The silkworm BmNPV-susceptible strain P50 (LC_{50} = 1.03 × 10^5) and BmNPV-resistant strain BC9 (LC_{50} = 5.90 × 10^7) were maintained in the Anhui International Joint Research and Development Centre of Sericulture Resources Utilisation, Anhui Agricultural University, Hefei, China. The first-to third-instar larvae were reared using a fresh artificial diet at 26 ± 1 °C, 75 ± 5% relative humidity, and 12 h day/night cycles. The rearing temperature for the fourth-to fifth-instar was adjusted to 24 ± 1 °C, with the same humidity and photoperiod described above. The BmNPV T3 strain was kept in our laboratory and was used for oral infection. BV was provided by Jiangsu University, Zhenjiang, China and was used for the virus overlay assay.

2.2. Immune challenge and tissues isolation

The overnight cultured *P. aeruginosa* and *S. aureus* cells were harvested by centrifugation at 7500 g for 10 min. The bacteria were washed three times and then suspended with sterilised 0.85% NaCl. The first day of the fifth-instar larvae were used for the bacterial infection. The infection groups were injected with 10 μL of *P. aeruginosa* or *S. aureus* cells (1×10^7) sterilised with 0.85% NaCl, and the control groups were injected with 10 μL of sterilised 0.85% NaCl. BmNPV oral infection was performed according to previous protocol (Wang et al., 2016). In brief, on the first day of the fifth instar, all larvae were starved for 24 h, and then were administrated 10 μL of BmNPV suspended in 0.85% NaCl. Ten larvae in each group were dissected to collect the hemolymph and midgut at 6, 12, 24 and 48 h post-infection. Each treatment was conducted in three biological replicates. The obtained tissue samples were stored in TRIzol reagent (Invitrogen, Grand Island, NY, USA) at −80 °C.

2.3. RNA isolation, cDNA synthesis and RT-qPCR analysis

Total RNA from different tissues was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The quality, purity, and concentration of RNA were measured at an absorbance ratio of $A_{260/280}$ and $A_{260/230}$ using a NanoDrop 2000 spectrophotometer (Thermo Scientific, New York, USA) and RNA integrity was checked by 1.0% agarose gel electrophoresis. The first strand cDNA was synthesised using PrimeScript RT reagent kit with gDNA Eraser based on the manufacturer's instructions (TaKaRa, Dalian, China). In brief, the concentration of each RNA sample was adjusted to 1 μg/μL with nuclease-free water, and total RNA was reverse-transcribed in a 20 μL reaction system. The qualified cDNA was stored at −20 °C for later use.

The relative expression level of two ferritin subunits was determined by reverse transcription quantitative PCR (RT-qPCR). Primers used for RT-qPCR are shown in Table 1. The RT-qPCR reaction was carried out in a 25 μL reaction mixture containing SYBR Premix Ex Taq (TaKaRa) according to the manufacturer's instructions. The reaction was performed in a CFX96TM Real-Time System (Bio-Rad, Hercules, CA, USA). The thermal cycling profile consisted of an initial denaturation at 95 °C for 30 s and 40 cycles at 95 °C for 5 s and 60 °C for 30 s. All assays were performed in triplicate. Relative expression levels were calculated using the $2^{-\triangle\triangle Ct}$ method (Yu et al., 2007). In the present study, *B. mori* glceraldehyde-3-phosphate dehydrogenase (*BmGAPDH*) was chosen as the reference gene. The statistical analyses was conducted using an analysis of variance (ANOVA) and a least significant difference (LSD) posteriori test with SPSS software (IBM, USA).

2.4. Iron overload and extraction of ferritin protein

The first day of the fifth-instar larvae were fed fresh mulberry spread with 10 μL 10% ferric chloride (Sangon Biotech, Shanghai, China) and the control group was administrated fresh mulberry treated with 10 μL 0.85% NaCl. A total of 120 P50 larvae was used and were divided equally into three independent experiments (that is, 40 larvae per group). In each group, 10 larvae were dissected to collect the midgut and hemolymph at 12, 24, 48 and 72 h respectively, and then all

Table 1
Primers used in this study.

Gene names	Primers (5′-3′)	Length (bp)
BmFerHCH	F: ACTTAGCCATGGGGGCTTAC R: TTACGGAGCCTGTCAGCTTT	193
BmFerLCH	F: CGTTGTAGCGAACGAACTGA R: TCCCAACCCTCTTAGTGACG	149
BmGADPH	F: CCGCGTCCCTGTTGCTAAT R: CTGCCTCCTTGACCTTTTGC	106