The Influence of Challenge on Cathepsin B and D Expression Patterns in the Silkworm *Bombyx mori* L.

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(Received 30 June 2011; Accepted 05 September 2011)

Cathepsins are well-characterized proteases that are ubiquitously expressed in lysosomes. Previous work revealed that *Bombyx mori* cathepsins B and D are expressed in the fat body and undergo decomposition during larval-pupal metamorphosis. Quantitative RT-PCR was performed to detect cathepsin gene expression at the transcription level when challenged by *B. mori* nuclear polyhedrosis virus (BmNPV), temperature and hormones (20-hydroxyecdysone (20E) and juvenile hormone analogue (JHA)). mRNAs encoding cathepsins B and D were significantly enhanced after the larvae were infected with BmNPV, and the peak of the induction appeared at 1 day before spinning. This attenuated the inducing effect on cathepsin expression caused by infection. Temperature shock induced cathepsin expression at the later stage of the 5th instar, and transcription levels varied with development stage and temperature. Cathepsin B and D mRNA expression in the fat body were significantly induced by JHA at the day before spinning, and with 20E, the expression reached a peak at the last day of the 5th instar. Cathepsin B and D mRNA expression exhibited detectable changes post-treatment, without significant differences between or among the hormone concentrations.

Key words: *Bombyx mori*, Cathepsin B and D, Expression, BmNPV, Hormones, Temperature

Introduction

Cathepsins are ubiquitous lysosomal enzymes and can be divided into three subgroups according to their active-site amino acid (i.e., cysteine: B, C, H, F, K, L, O, S, V, W cathepsins; aspartic acid: D and E cathepsins; and serine: G cathepsins) (Duve, 1983). Proteases of the cathepsin family are among the most studied lysosomal hydrolases that degrade proteins in lysosomes at an acidic pH. Apart from their function in general protein turnover, housekeeping, and protein processing, knock-out experiments have revealed that cathepsins perform specific functions in mammals, such as neovascularization of endothelial progenitor cells (Urbich *et al.*, 2005), antigen presentation (Nakagawa *et al.*, 1998), cell growth (Saftig *et al.*, 1995), tissue homeostasis (Nakanishi *et al.*, 2001) and apoptosis (Fehrenbacher and Jaattela, 2005). Cathepsin gene transcriptional up-regulation has been reported in immune-stimulated *Drosophila* and *Glossina morsitans morsitans* (De Gregorio *et al.*, 2001; Attardo *et al.*, 2006). Several lines of evidence suggest that cathepsins are involved in insect immune responses (Zhong *et al.*, 2001; Ahn and Keyan, 2009).

Cathepsin D is a proteolytic enzyme of lysosomes, and like most of the lysosomal enzymes, it usually occurs as a glycoprotein. This protease has acidic pH optima ranging from pH 3 to 5 and relies on two aspartic acid residues for catalysis. In insects, cathepsin D is implicated in physiological processes such as vitellogenin production and degradation in mosquitoes (Cho and Raikhel, 1992), digestion (Silva and Xavier-Filho, 1991; Brunelle *et al.*, 1999), fat body histolysis in *Ceratitis capitata* (Rabossi *et al.*, 2004), and programmed cell death for the larval-pupal transformation in *Bombyx mori* (Gui *et al.*, 2006). The cathepsin D enzyme also acts in blood meal digestion in ectoparasitic mites and ticks (Boldbaatar *et al.*, 2006).

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Cathepsin B is a cysteine proteinase normally localized to lysosomes. It has been reported in numerous insects and can take part in physiological processes such as ontogenesis, and tissue differentiation through its non-specific proteolytic function (Carnevali *et al.*, 2001; Shompole and Jasmer, 2001), as well as histolysis and histogenesis during metamorphosis (Lee *et al.*, 2009). Cathepsin B participates in decomposition during the larval-pupal metamorphosis of *B. mori* (Shiba *et al.*, 2001; Xu and Kawasaki, 2001) and in the decomposition of the larval fat body during metamorphosis of the flesh fly, *Sarcophaga peregrina* (Takahashi *et al.*, 1993).

Our recent study of *B. mori* metamorphosis showed that both cathepsin B and D are involved in fat body histolysis during larval-pupal transformation (Gui *et al.*, 2006; Lee *et al.*, 2009). Nonetheless, the biological significance of these enzymes projects far beyond metamorphosis. Because cathepsins act in insect immunity, we posed the hypothesis that cathepsins act in insect responses to other challenges as well. In this study, we investigated the expression patterns of *B. mori* cathepsin B and D during the last instar phase when challenged by *B. mori* nuclear polyhedrosis virus (BmNPV), temperature and insect hormones (20-hydroxyecdysone (20E) and juvenile hormone analogue (JHA) using fluorescence quantitative real-time PCR.

Materials and Methods

Insects

The larvae of the silkworm, *B. mori*, used in this study were F1 hybrid Suzhen $\times$ Chunguang and were supplied by the Sericultural Research Institute, Chinese Academy of Agricultural Science, Zhenjiang city, China. Silkworms were reared on fresh mulberry leaves at 25°C with 75 $\pm$ 5% relative humidity and a 12-h light:12-h dark photoperiod.

Treatment of *B. mori* larvae

1. *B. mori* nuclear polyhedrosis virus (BmNPV) infection

Two hundred larvae for each treatment were used. BmNPV (5×10^7 polyhedra/ml and 10×10^7 polyhedra/ml) was administered with fresh mulberry leaves to fifth instar larvae from 0 h to 24 h.

2. Temperature treatment

For temperature treatment, *B. mori* larvae were divided into three groups and exposed to three temperatures, 22°C, 25°C and 28°C, from day 1 of the fifth instar to spinning, typically about 8 days.

3. Hormone exposure

Hormonal exposure treatment was applied as described previously (Gui *et al.*, 2009). 20-Hydroxyecdysone (20E, Sigma) was dissolved in distilled water for stock concentration of 1 mg/ml. Concentrations of 0.5 μ g/ μ l, 1.0 μ g/ μ l and 2.0 μ g/ μ l of 20E were injected into *B. mori* larvae on day 3 of the fifth instar. Concentrations of 2 μ g/ μ l and 4 μ g/ μ l of a JHA, fenoxycarb (Sankyo, Japan), dissolved in acetone were applied topically to larvae on day 3 of the fifth instar with a micropipette along the dorsal midline.

Tissue collection and RNA extraction

The fat bodies from all treated larvae were collected on ice at 1-day intervals post-treatment and washed twice with PBS (140 mM NaCl, 27 mM KCl, 8 mM Na₂HPO₄ and 1.5 mM KH₂PO₄, pH 6.5). The homogenates were centrifuged at 10,000 $\times$ g for 20 min, and the pellets were discarded. Total RNA was extracted from fat bodies using TRIzol (GENERAY, China) according to the manufacturer's instructions. Total RNA was dissolved in water treated with diethyl pyrocarbonate (DEPC), and the concentration was measured using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). DNA within RNA samples was digested with RNase-free DNase I. The quality of RNA was analyzed on 1.0% agarose gels.

Reverse transcription and primer design

RT-PCR was performed as previously described (Zou *et al.*, 2011). The first strand of cDNA was synthesized using PrimerScriptTM RT Enzyme Mix I Reverse Transcriptase following the manufacturer's instructions (TaKaRa Code: DRR037A, China). Briefly, reverse transcription was carried out in 0.65-ml RNase-free tubes under optimized conditions in a DNA Thermal Cycler 480 (PerkinElmer). Equal amounts of total RNA (500 ng) from each sample were used for these reactions. Each reaction was performed at 37°C for 15 min and 85°C for 5 s in a total volume of 10 μ l containing 2 μ l 5 $\times$ PrimeScriptTM Buffer, 0.5 μ l PrimeScriptTM RT Enzyme Mix I, 0.5 μ l of 50 mol/l OligodT primer, 1 μ l total RNA and RNase-free H₂O. The following specific primers for cathepsins B and D were designed and prepared according to the available information for these specific genes (Gui *et al.*, 2006; Lee *et al.*, 2009). The primers for *B. mori* cathepsin B (5'- AACATCACGTACCCGGAACA -3' and 5'- CGCGGATGTGGTCTTCGTCT -3') amplified a 159-bp product (642–800); the primers for *B. mori* cathepsin D (5'- ATTTCTCATAAGCAAGTCGC -3' and 5'- AGTGGGTTCTCGCAGTTTT -3') amplified a 146-bp product (97–242). The primers for the housekeeping gene actinA3 (5'- ATGGGACAGAAGGACTCGTA -3' and 5'- GACACGCAGCTCATTGTAGA -3') amplified a

150-bp product (2574–2723) and were used as an internal control for comparison with target genes at all time points. Finally, the amplified genes were resolved by electrophoresis and visualized using ethidium bromide staining. (The GenBank accession numbers of the cathepsin B, cathepsin D and actinA3 cDNA are AB045595.1, AY297160.1 and U49854.1, respectively.)

Quantitative real-time RT-PCR

To compare the influence of hormones (JHA and 20E), BmNPV and temperature on the transcript levels of cathepsin B and D, the *B. mori* actin A3 gene was used as the control gene. Real-time PCR was performed in a total reaction volume of 20 μ l, containing 10 μ l of 1 $\times$ SYBR GREEN PCR mix (TaKaRa Code: DRR041A), 1 μ l 1:10-diluted cDNA templates and 1 μ l of each primer (1 μ mol/l actinA3 and 10 μ mol/l cathepsin B and cathepsin D), according to the manufacturer's instructions. Reaction conditions were as follows: 94°C for 3 min and 40 cycles of 94°C for 5 s, 60°C for 10 s, and 72°C for 10 s. To confirm the amplification of specific products, melting curve cycles continued with the following parameters: 95°C for 1 min, 55°C for 1 min, 80 cycles of 55°C for 10 s and a 0.5°C increase per cycle. Amplification and detection of SYBR® Green were performed using MyiQ (Bio-Rad, Hercules, CA, USA). Data were collected as C_T (PCR cycle number at which fluorescence was detected above the threshold and decreased linearly with increasing target quantity) using the MyiQ optical system software version 1.0 (Bio-Rad). All experiments were performed in triplicate; each sample was run in triplicate on PCR plates, and data were pooled. The C_T of each sample was used to calculate the ΔC_T values (target gene C_T subtracted from actinA3 C_T). The respective mRNA expression data were first normalized to actinA3 expression to compensate for any difference in reverse transcriptase efficiency. The relative fold change of the mRNA expression of a gene, compared to control, was automatically analyzed by the Opticon Monitor 3, and efficiency was corrected for according to the method of Schefe (2006).

Statistical analysis

All data were compared by one-way analysis of variance (ANOVA) followed by Tukey's studentized test when significant differences were found at P 0.05 (SAS, 1997). Differences between samplings were considered statistically significant at a probability less than 5% and marked in figures.

Results

Cathepsin B and D expression after BmNPV challenge

As shown in Fig. 1, the relative expression of mRNA

Fig. 1. Expression profile of cathepsin B mRNA in *B. mori* infected by *Bombyx mori* nuclear polyhedrosis virus (BmNPV). Silkworm larvae on the 1st day of the fifth instar were infected with BmNPV (5×10^7 and 10×10^7). Larval fat bodies were collected at 1-day intervals post-treatment. Each experiment was performed in triplicate. Cathepsin B mRNA expression values were normalized to actin A3 expression and are shown as the mean $\pm$ S.E. ($n = 3$).

Fig. 2. Expression profile of cathepsin D mRNA in *B. mori* infected by *Bombyx mori* nuclear polyhedrosis virus (BmNPV). Silkworm larvae on the 1st day of the fifth instar were infected with BmNPV (5×10^7 and 10×10^7). Larval fat bodies were collected at 1-day intervals post-treatment. Each experiment was performed in triplicate. Cathepsin D mRNA expression values were normalized to actin A3 expression and are shown as the mean $\pm$ S.E. ($n = 3$).

encoding cathepsin B was high at day 1 post-infection, then decreased on the next day, and reached its highest level on day 2 before death on day 7. The highest cathepsin B expression in the high BmNPV concentration (10×10^7 polyhedra/ml) group appeared at an earlier time point, compared to results with the lower BmNPV concentration (5×10^7 polyhedra/ml) group (Fig. 1). However, cathepsin D expression reached its highest level on day 2 before larval death on day 7 in both BmNPV concentration groups, which is similar to cathepsin B (Fig. 2).

Cathepsin B and D expression after temperature shock

The larval span at 25°C is 7 days, while higher temperatures shortened the larval span to 6 days, and lower temperatures increased the larval period to 9 days. Cathepsin B and D expression varied with development and temperature shock. High temperatures induced cathepsin B expression at both the earlier and later stage, while the moderate and lower temperatures induced cathepsin B expression significantly at the later stage of the 5th instar (Fig. 3). Temperature shock induced increased cathepsin D from 1-day post-treatment, and the expression reached its peak at day 2 before spinning. The influence of the

Fig. 3. Expression profile of cathepsin B mRNA in *B. mori* challenged by temperature. Silkworm larvae were reared at different temperature conditions (22°C, 25°C or 28°C). Larval fat bodies were collected every 24 h. Each experiment was performed in triplicate. Cathepsin B mRNA expression values were normalized to actin A3 expression and are shown as the mean $\pm$ S.E. (n = 3).

Fig. 4. Expression profile of cathepsin D mRNA in *B. mori* challenged by temperature. Silkworm larvae were reared at the different temperature conditions (22°C, 25°C or 28°C). Larval fat bodies were collected every 24 h. Each experiment was performed in triplicate. Cathepsin D mRNA expression values were normalized to actin A3 expression and are shown as the mean $\pm$ S.E. (n = 3).

experimental thermal treatments for cathepsin D expression showed a consistent trend (Fig. 4), i.e. increasing to late stage and reach a peak at 1 - 2 days before the end of 5th instar. Comparing the cathepsins, cathepsin B expression is nearly hundred times greater than cathepsin D expression.

Cathepsin B and D expression after hormone (20E and JHA) challenge

The results showed that JHA delayed the larval duration 1-2 days, while 20E shortened the larval span by 1 day. There was abundant constitutive expression of cathepsins B and D at the mRNA level (Figs. 5 and 6). After larvae were treated with JHA, cathepsin B and D mRNA expression in the fat body was significantly induced; the highest expression appeared at the day before spinning (day 9 of the last instar phase), with a weak decrease in expression at the spinning day (Figs. 5A and 6A). Larvae treated with 20E also showed enhanced expression of cathepsins B

Fig. 5. Expression profile of cathepsin B mRNA in *B. mori* treated with hormones JHA (A) and 20E (B). Silkworm larvae on the 3rd day of the fifth instar were exposed to JHA (2.0 mg/ml and 4.0 mg/ml) or 20E (0.5 mg/ml, 1.0 mg/ml and 2.0 mg/ml). Larval fat bodies were collected at 1-day intervals post-treatment. Each experiment was performed in triplicate. Cathepsin B mRNA expression values were normalized to actinA 3 expression and are shown as the mean $\pm$ S.E. (n = 3).

Fig. 6. Expression profile of cathepsin D mRNA in *B. mori* treated with hormones JHA (A) and 20E (B). Silkworm larvae on the 3rd day of the fifth instar were exposed to JHA (2.0 mg/ml and 4.0 mg/ml) or 20E (0.5 mg/ml, 1.0 mg/ml and 2.0 mg/ml). Larval fat bodies were collected at 1-day intervals post-treatment. Each experiment was performed in triplicate. Cathepsin D mRNA expression values were normalized to actin A3 expression and are shown as the mean $\pm$ S.E. ($n = 3$).

and D, the expression reached a peak at the last day of the 5th instar (Fig. 5B and 6B). The mRNA expression of cathepsins B and D exhibited detectable changes post-treatment, and no significant differences were observed between the concentrations used (2 μ g/ μ l and 4 μ g/ μ l of JHA, 0.5 μ g/ μ l, 1.0 μ g/ μ l and 2.0 μ g/ μ l of 20E) (Fig. 5A and 6A).

Discussion

Cathepsins are expressed in a variety of cells, and cathepsins B and D are among the most abundant lysosomal proteases (Turk *et al.*, 2000). Physiologically, cathepsins degrade proteins nonspecifically inside lysosomes. Expression of cathepsin B and D in insects occurs mostly in fat bodies, oocytes, and hemocytes (Cho *et al.*, 1999; Snigirevskaya *et al.*, 1997).

The larval fat body of *S. peregrina* is degraded during

the larval–pupal metamorphosis (Takahashi *et al.*, 1993). Because *B. mori* undergoes complete metamorphosis, its larval fat body, midgut and silk gland degenerate and are replaced by adult tissues and structures. The involvement of a cathepsin B-like proteinase in fat bodies of the *H. armigera* during pupal–adult metamorphosis has been demonstrated (Yang *et al.*, 2006). Kurata *et al.* (1992) suggested that a *S. peregrina* cathepsin B-like proteinase could be released from hemocytes when hemocytes contacted the fat body and further degrade the basement membrane of the fat body. Xu and Kawasaki (2001) observed many hemocytes attaching to the fat body tissues during larval–pupal metamorphosis in *B. mori*. Hemocytes distributed in such tissues, together with the increased expression of cathepsin B-like proteinase in hemocytes before larva pupation, played a key role in fat body and midgut histolysis during the larval–pupal metamorphosis of *H. armigera* (Yang *et al.*, 2007).

Previously, we showed the expression profile of cathepsin B (Lee *et al.*, 2009) and D (Gui *et al.*, 2006) in the fat body of the silkworm *B. mori* during development and after RNA interference (RNAi)-mediated inhibition. Cathepsin B is induced by 20-OH-ecdysone and is expressed in the fat body of *B. mori* during molting and the larval–pupal and pupal–adult transformations, and its expression leads to programmed cell death. In this study, our results showed relatively higher levels of cathepsin B and D expression after challenge by hormones (JHA and 20E). A high level of expression of cathepsin B and D were observed in the fat body of *B. mori* during an arrest of the larval–pupal transformation. We infer that cathepsin B and D participate in fat body apoptosis during *B. mori* metamorphosis. Insect hemocytes play an important role in larval–pupal metamorphosis and the increase in plasmatocytes and granulocytes before larvae pupation increases the expression of cathepsin B-like proteinase in *S. peregrina* (Kurata *et al.*, 1992), cathepsin B in *B. mori* (Xu and Kawasaki, 2001), and cathepsin B-like proteinase in *H. armigera* (Yang *et al.*, 2007).

Cathepsins occur in lysosomes and are also associated with the plasma membrane or are secreted, indicating their role in the digestion of the extracellular matrix (Sloane *et al.*, 1994; Heidtmann *et al.*, 1993). Cathepsins are involved in insect immune responses. In the flesh fly *S. peregrina*, a 26/29-kDa protease is specifically released by hemocytes after an immune challenge (Saito *et al.*, 1992), and in *Drosophila*, cathepsin L protein is induced in the hemolymph of adults after fungal infection (Levy *et al.*, 2004). Transcriptional up-regulation of cathepsin has been reported in immune-stimulated *Drosophila* and *G. morsitans morsitans* (De Gregorio *et al.*, 2001; Attardo *et al.*, 2006). In this study, we determined that the expression

of cathepsin B and D was modulated at the transcription level by BmNPV infection. The relative expression of cathepsin B and D was highest on day 2 before death. Serbielle *et al.* (2009) studied the role of polydnavirus cystatins in the host-parasitoid interaction to characterize *Manduca sexta* cathepsins (MsCath1 and MsCath2) and revealed that MsCath1 and MsCath2 are transcriptionally down-regulated in the course of parasitism. Their results also show that parasitism induces a general increase of cysteine protease activity.

Temperature-induced cathepsin B and D expression increased at the later stage of the 5th instar. Different temperature conditions showed a consistent cathepsin expression trend. We infer that cathepsin B plays a more important role during larval-pupal transformation than cathepsin D.

Acknowledgement

This work was supported by the National Natural Science Foundation of China grant No. 30972144.

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