IJIE Int. J. Indust. Entomol. 45(2) 43-48 (2022)
ISSN 1598-3579, http://dx.doi.org/10.7852/ijie.2022.45.2.43

ISSN 1598-3579 (Print)
ISSN 2586-4785 (Online)

Role of the prophenoloxidase-activating system in the innate immune response and cuticular melanization in the silkworm

Kwang Sik Lee*

College of Natural Resources and Life Science, Dong-A University, Busan 49315, Republic of Korea

Abstract

Bombyx mori is a representative industrial insect and is used in silk production. Additionally, it serves as an insect model in molecular studies. To date, various molecular studies on its physiological characteristics, including the innate immune response and cuticular melanization, have been conducted. The melanization, including cuticular melanization, in insects is controlled by the prophenoloxidase-activating system, which is also involved in their innate immune response. In this review, to better understand the molecular mechanisms underlying the prophenoloxidase-activating system in the silkworm, the roles of five biomolecules, namely tyrosine hydroxylase, prophenoloxidase-activating enzyme, phenoloxidase, serine protease homolog, and immulectin, are discussed.

Int. J. Indust. Entomol. 45(2), 43-48 (2022)

Received : 02 Dec 2022
Revised : 19 Dec 2022
Accepted : 22 Dec 2022

Keywords:
Silkworm,
Innate immune response,
Cuticular melanization,
Prophenoloxidase-activating system

Introduction

Melanin is one of the major components of the innate immune response and cuticular hardening in insects and is formed through the regulation of the prophenoloxidase-activating system (Cerenius and Söderhäll, 2004; Cerenius *et al*., 2008). This system consists of a series of successive proteolytic enzyme reactions that eventually generate the active phenoloxidase (Cerenius and Söderhäll, 2004). Microbial infections activate the serine proteases that activate theses proteolytic, prophenoloxidase-activating enzymes (Ashida and Brey, 1995; Jiang and Kanost, 2000). Additionally, the proteolytic prophenoloxidase-activating enzymes cleavage of prophenoloxidase and change into the active phenoloxidase by prophenoloxidase-activating enzymes, resulting in the production of quinones that form melanin, which is used to kill microbes (Cerenius and Söderhäll, 2004). Since excessive melanin can damage the host, the prophenoloxidase-activating system is tightly controlled by serine protease inhibitors (serpins) (Cerenius *et al*., 2008). Melanin is generated from the amino acid L-tyrosine though an oxidative process (Riley, 1997). In this process, first, tyrosine is hydrolyzed into dopa by tyrosine hydroxylase, an enzyme that responds to cuticular melanization (Futahashi and Fujiwara, 2005; Gorman *et al*., 2007; Gorman and Arakane, 2010). The next enzyme, dopa decarboxylase, converts dopa into dopamine (Wright, 1987; Hopkins and Kramer, 1992). The activated phenoloxidase induces the oxidation of dopa or dopamine to produce quinones, leading to melanin synthesis (Hiruma and Riddiford, 2009).

In insect cuticles, phenoloxidase-mediated melanin synthesis serves toward cuticular hardening and tanning through protein cross-linking (Söderhäll and Cerenius, 1998; Anderson, 2005). In addition to cuticular tanning, melanin causes cuticular hardening by cross-linking proteins of the extracellular matrix (Kramer and

***Corresponding author.**
Kwang Silk Lee, Ph.D.
College of Natural Resources and Life Science, Dong-A University, Busan 49315, Republic of Korea
Tel: +82-51-200-5750
E-mail: leeks@dau.ac.kr

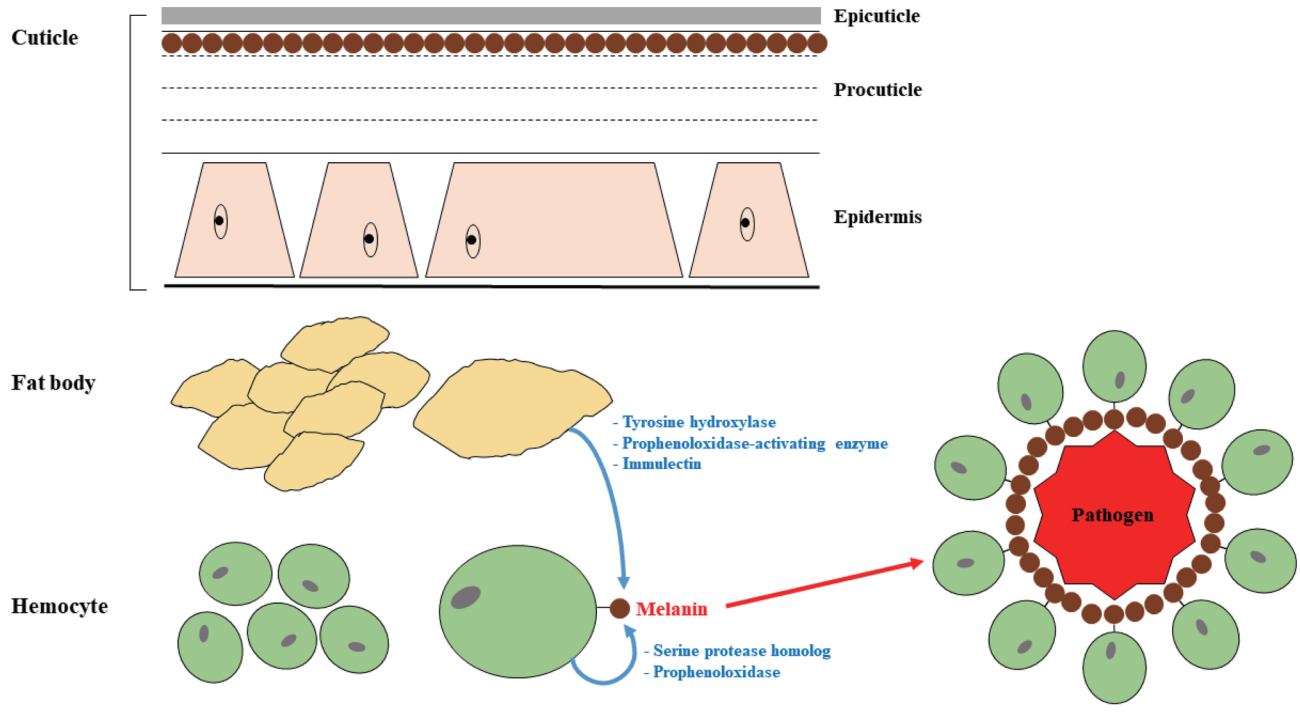

Fig. 1. Proposed model of the prophenoloxidase-activating system in the innate immune response.

Hopkins, 1987; Riley, 1997).

The signaling pathways involved in cuticular melanization have been described in several insects, such as *Tribolium castaneum* (Arakane *et al*., 2009), *Manduca sexta* (Hiruma and Riddiford, 2009), *B. mori* (Zhan *et al*., 2010), and *Papilio xuthus* (Futahashi and Fujiwara, 2005). These studies indicate that the prophenoloxidase-activating system plays an important role in the innate immune responses and cuticular melanization. This system is activated upon the binding of the pattern-recognition proteins in insect cells to microbial cell-wall components, such as ß-1,3-glucans, lipopolysaccharides, mannan, and peptidoglycans (Kanost *et al*., 2004; Lemaitre and Hoffmann, 2007). In addition, the pattern-recognition receptor immulectin, which binds to the surface carbohydrates on pathogens, may act as an anchor on the pathogen surface during the formation of the prophenoloxidase-activating complex. This complex consists of immulectin, serine protease homolog, prophenoloxidase-activating enzyme, and prophenoloxidase (Yu *et al*., 2003). Interestingly, the prophenoloxidase-activating system is also activated in cuticular melanization, without the involvement of microbial cell-wall components. Data from insect studies suggest that tyrosine hydroxylase, prophenoloxidase-activating enzyme, phenoloxidase, serine protease homolog, and immulectin are important proteins in the prophenoloxidase-activating system (Yu *et al*., 2003; Cerenius and Söderhäll, 2004; Kanost *et al*., 2004; Gorman *et al*., 2007). This review discusses how these five proteins participate in the cuticular melanization of the pupae and how the cuticular melanization mechanistically differs from the innate immune response in the silkworm *Bombyx mori*.

Proteins involved in the prophenoloxidase-activating system

1. Tyrosine hydroxylase

Tyrosine hydroxylase constitutes the first enzyme in the prophenoloxidase-activating system and converts tyrosine into dopa. It is expressed in the fat body of insects (Gorman *et al*., 2007; Hashimoto *et al*., 2008), including the fat body of silkworm pupae (Lee *et al*., 2015), upon microbial infection. The phenoloxidase substrate dopamine can be released into hemolymph and can be used to kill the infecting microbes after an oxidation reaction (Gorman *et al*., 2007).

Conversion of tyrosine into dopa by tyrosine hydroxylase is also the first step in cuticular melanization (True *et al*., 1999; Futahashi and Fujiwara, 2005). Tyrosine hydroxylase is

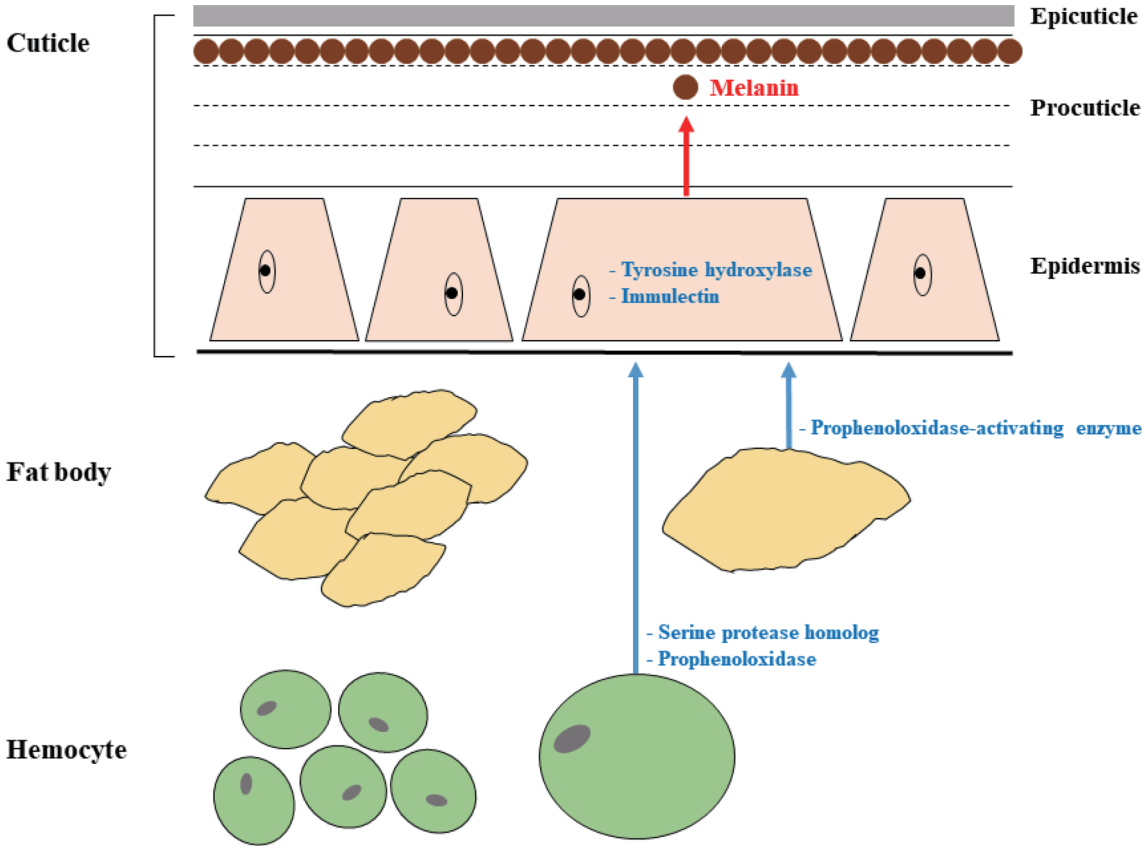

Fig. 2. Proposed model of the prophenoloxidase-activating system in the cuticular melanization.

expressed in the epidermis of *B. mori* during the larval-pupal and pupal-adult transformations (transitions from the late pre-pupal stage to the early pupal stage, and from the late pupal stage to the early adult stage, respectively) (Lee *et al*., 2015). A tyrosine hydroxylase inhibitor or RNA interference of tyrosine hydroxylase delays the cuticular melanization of silkworm pupae and suppresses their cuticular tanning and hardening (Gorman and Arakane, 2010; Liu *et al*., 2010; Lee *et al*., 2015). Thus, tyrosine hydroxylase expression is spatiotemporally regulated during the innate immune response and cuticular melanization in *B. mori*.

2. Prophenoloxidase-activating enzyme and prophenoloxidase

The functional role of phenoloxidases in cuticular melanization and innate immune response have been reported in many insects (Cerenius and and Söderhäll, 2004; Kanost *et al*., 2004; Andersen, 2005). Prophenoloxidase-activating enzyme converts the inactive prophenoloxidase into the active phenoloxidase, which then catalyzes the production of quinones. These enzymes are essential for the prophenoloxidase-activating system (Ashida and Brey, 1995; Jiang and Kanost, 2000). When the silkworm is infected with *Escherichia coli*, the mRNA of prophenoloxidase-activating enzyme is up-regulated in the fat body, and the protein is detected in hemocytes. Prophenoloxidase mRNA is also up-regulated in hemocytes. RNA interference of prophenoloxidase-activating enzyme reduces the phenoloxidase activity in hemolymph upon *E. coli* infection (Zou *et al*., 2015). Thus, prophenoloxidase-activating enzyme is essential for the phenoloxidase activation in the innate immune response.

Silkworm pupae change their color patterns from milky white to dark brown during the early pupal stage. The mRNA of *B. mori* prophenoloxidase-activating enzyme is expressed in the fat body during the early pupal stage, and the protein is detected in the fat body and cuticle (Zou *et al*., 2015). Previous studies have reported that prophenoloxidase is sythesized in hemolymph and then translocated to the cuticle (Ashida and Brey, 1995; Asano ans Ashida, 2001a, 2001b). The mRNAs of *B. mori* phenoloxidases are expressed in hemocytes during

the pre-pupal stage, and the proteins are detected in the cuticle (Zou *et al*., 2015). These results support the translocation of prophenoloxidase-activating enzyme and prophenoloxidase from hemocytes to cuticle for the pupal cuticular melanization.

3. Serine protease homolog

Serine proteases, including serine protease homolog, act as the activators of the prophenoloxidase-activating enzyme (Ashida and Brey, 1995; Jiang and Kanost, 2000). When the silkworm is infected with *E. coli*, serine protease homolog mRNA is up-regulated in hemocytes, and the corresponding protein is detected in its cleaved, active form in hemolymph (Lee *et al*., 2018). RNA interference of serine protease homolog decreases phenoloxidase activity, implying that serine protease homolog is necessary for the prophenoloxidase activity during infection (Lee *et al*., 2018).

Serine protease homolog mRNA is also expressed in hemocytes during the spinning and pre-pupal stages of the silkworm. Moreover, serine protease homolog protein is detected in its active form in the cuticle during the pre-pupal and early pupal stages (Lee *et al*., 2018). RNA interference of serine protease homolog delays the cuticular melanization in the pre-pupal stage (Lee *et al*., 2018). Thus, this protein is also essential for the propenoloxidase-activating system in cuticular melanization.

4. Immulectin

Pattern-recognition receptors mediate the innate immune response in insects and recognize the surface carbohydrates on pathogens (Kanost *et al*., 2004; Lemaitre and Hoffmann, 2007). C-type lectins are pattern-recognition receptors with carbohydrate-recognition domains (Dodd and Drickamer, 2001; Cambi *et al*., 2005). C-type lectin-S and immulectin, which contain a single and two carbohydrate-recognition domains, respectively, and C-type lectin-X, which has a complex domain structure, have been identified in the silkworm (Rao *et al*., 2015). Additionally, the targets and functions of these proteins have been reported (Koizumi *et al*., 1997; 1999a, 1999b; Watanabe *et al*., 2006; Takase *et al*., 2009; Kim and Jin, 2017). Immulectin from the silkworm can bind to fungi, yeast, and gram-positive and -negative bacteria (Kim and Jin, 2017). When the silkworm is infected with *E. coli*, immulectin mRNA is up-regulated in the fat body, and immulectin protein is detected in hemocytes. Additionally, immulectin mRNA is expressed in the cuticle during the pre-pupal stage, and the protein is detected in the cuticle during the pre-pupal and early pupal stages (Kim and Jin, 2017). RNA interference of immulectin decreases the melanin synthesis in the pupal cuticle (Kim and Jin, 2017). Thus, immulectin is involved in the innate immune response as well as cuticular melanization in *B. mori*.

Conclusion

In insects, the prophenoloxidase-activating system plays an important role not only in the melanization response of the innate immune system but also in the cuticular melanization during development. Here, we described how the key components of this system (tyrosine hydroxylase, prophenoloxidase-activating enzyme, phenoloxidase, serine protein homolog, and immulectin) differentially participate in the cuticular melanization and innate immune response in the silkworm *B. mori* (Fig. 1 and Fig. 2). First, tyrosine hydroxylase is expressed in the fat body to initiate the innate immune response or in the epidermis for cuticular melanization. Second, prophenoloxidase-activating enzyme is expressed in the fat body and then transported to hemocytes for either of these process. Third, phenoloxidases are expressed in hemocytes, and during cuticular melanization, they are transported to the cuticle. Fourth, serine protease homolog is expressed in hemocytes and likewise transported to the cuticle during cuticular melanization. Finally, upon infection, immulectin is expressed in the fat body and then transported to hemocytes and/or hemolymph for the innate immune response. In contrast, during development, immulectin is expressed in the epidermis and then transported to the cuticle for cuticular melanization. Elucidation of the mechanistic details underlying the prophenoloxidase-activating system priming the cuticular melanization and innate immunity in *B. mori* will further our understanding of the physiological roles of this system in insects.

References

Andersen SO (2005) Cuticular sclerotization and tanning. In: Gilbert LI, Iatrou K, Gill SS (Eds.), Comprehensive Molecular Insect Science vol. 4. Elsevier, Amsterdam, pp. 145–170.

Arakane Y, Lomakin J, Beeman RW, Muthukrishnan S, Gehrke SH, Kanost MR, *et al*. (2009) Molecular and functional analyses of amino acid decarboxylases involved in cuticle tanning in *Tribolium*

castaneum. J Biol Chem 284, 16584-16594.

Asano T, Ashida M (2001a) Transepithelially transported pro-phenoloxidase in the cuticle of the silkworm, *Bombyx mori*: identification of its methionyl residues oxidized to methionine sulfoxides. J Biol Chem 276, 11113–11125.

Asano T, Ashida M (2001b) Cuticular pro-phenoloxidase of the silkworm, *Bombyx mori*: purification and demonstration of its transport from hemolymph. J Biol Chem 276, 11100–11112.

Ashida M, Brey PT (1995) Role of the integument in insect defense: pro-phenol oxidase cascade in the cuticular matrix. Proc Natl Acad Sci U. S. A. 92, 10698–10702.

Cambi A, Koopman M, Figdor CG (2005) How C-type lectins detect pathogens. Cell Microbiol 7, 481–488.

Cerenius L, Söderhäll K (2004) The prophenoloxidase-activating system in invertebrates. Immunol Rev 198, 116–126.

Cerenius L, Lee BL, Söderhäll K (2008) The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol 29, 263–271.

Dodd RB, Drickamer K (2001) Lectin-like proteins in model organisms: implications for evolution of carbohydrate-binding activity. Glycobiology 11, 71R–79R.

Futahashi R, Fujiwara H (2005) Melanin-synthesis enzymes coregulate stage-specific larval cuticular markings in the swallowtail butterfly, *Papilio xuthus*. Dev Genes Evol 215, 519–529.

Gorman MJ, An C, Kanost MR (2007) Characterization of tyrosine hydroxylase from *Manduca sexta*. Insect Biochem Mol Biol 37, 1327–1337.

Gorman MJ, Arakane Y (2010) Tyrosine hydroxylase is required for cuticle sclerotization and pigmentation in *Tribolium castaneum*. Insect Biochem Mol Biol 40, 267–273.

Hashimoto K, Yamano Y, Morishima I (2008) Induction of tyrosine hydroxylase gene expression by bacteria in the fat body of eri-silkworm, *Samia cynthia ricini*. Comp Biochem Physiol B 149, 501–506.

Hiruma K, Riddiford LM (2009) The molecular mechanisms of cuticular melanization: the ecdysone cascade leading to dopa decarboxylase expression in *Manduca sexta*. Insect Biochem Mol Biol 39, 245–253.

Hopkins TL, Kramer KJ (1992) Insect cuticle sclerotization. Ann Rev Entomol 37, 273-302.

Jiang H, Kanost MR (2000) The clip-domain family of serine proteinases in arthropods. Insect Biochem Mol Biol 30, 95–105.

Kanost MR, Jiang H, Yu ZQ (2004) Innate immune responses of a lepidopteran insect, *Manduca sexta*. Immunol Rev 198, 97–105.

Kim BY, Jin BR (2017) The dual roles of *Bombyx mori* immulectin in cuticular melanization and innate immunity. J Aisa-Pac Entomol 20, 761–766.

Koizumi N, Morozumi A, Imamura M, Tanaka E, Iwahana H, Sato R (1997) Lipopolysaccharide-binding proteins and their involvement in the bacterial clearance from the hemolymph of the silkworm *Bombyx mori*. Eur J Biochem 248, 217–224.

Koizumi N, Imai Y, Morozumi A, Imamura M, Kadotani T, Yaoi K, *et al*. (1999a) Lipopolysaccharide-binding protein of *Bombyx mori* participates in a hemocyte-mediated defense reaction against gram-negative bacteria. J Insect Physiol 45, 853–859.

Koizumi N, Imamura M, Kadotani T, Yaoi K, Iwahana H, Sato R (1999b) The lipopolysaccharide-binding protein participating in a hemocyte nodule formation in the silkworm *Bombyx mori* is a novel member of the C-type lectin superfamily with two different tandem carbohydrate-recognition domains. FEBS Lett 443, 139–143.

Lee KS, Kim BY, Jin BR (2015) Differential regulation of tyrosine hydroxylase in cuticular melanization and innate immunity in the silkworm *Bombyx mori*. J Asia-Pac Entomol 18, 765–770.

Lee KS, Kim BY, Choo YM, Jin BR (2018) Dual role of the serine protease homolog BmSPH-1 in the development and immunity of the silkworm *Bombyx mori*. Dev Comp Immunol 85, 170–176.

Lemaitre B, Hoffmann J (2007) The host defense of *Drosophila melanogaster*. Annu Rev Immunol 25, 697–743.

Liu C, Yamamoto K, Cheng TC, Kadono-Okuda K, Narukawa J, Liu SP, *et al*. (2010) Repression of tyrosine hydroxylase is responsible for the sex-linked chocolate mutation of the silkworm, *Bombyx mori*. Proc Natl Acad Sci U. S. A. 107, 12980–12985.

Rao XJ, Shahzad T, Liu S, Wu P, He YT, Sun WJ, *et al*. (2015) Identification of C-type lectin-domain proteins (CTLDPs) in silkworm *Bombyx mori*. Dev Comp Immunol 53, 328–338.

Riley PA (1997) Melanin. Int J Biochem Cell Biol 29, 1235–1239.

Kramer KJ, Hopkins TL (1987) Tyrosine metabolism for insect cuticle tanning. Arch Insect Biochem Physiol 6, 279–301.

Takase H, Watanabe A, Yoshizawa Y, Kitami M, Sato R (2009) Identification and comparative analysis of three novel C-type lectins from the silkworm with functional implications in pathogen recognition. Dev Comp Immunol 33, 789–800.

True JR, Edwards KA, Yamamoto D. Carroll SB (1999) Drosophila wing melanin patterns from by vein-dependent elaboration of enzymatic prepatterns. Curr Biol 9, 1382–1391.

Watanabe A, Miyazawa S, Kitami M, Tabunoki H, Ueda K, Sato R (2006) Characterization of a novel C-type lectin, *Bombyx mori* multibinding protein, from the *B. mori* hemolymph: mechanism of wide-range microorganism recognition and role in immunity. J

Immunol 177, 4594–4604.

Wright TRF (1987) The genetics of biogenic amine metabolism, sclerotization, and melanization in *Drosophila melanogaster*. Adv Genet 24, 127–222.

Yu XQ, Jiang H, Wang Y, Kanost MR (2003) Nonproteolytic serine proteinase homologs are involved in prophenoloxidase activation in the tobacco hornworm, *Manduca sexta*. Insect Mol Biol 33, 197–208.

Zhan S, Guo Q, Li M, Li M, Li J, Miao X, *et al*. (2010) Disruption of an N-acetyltransferase gene in the silkworm reveals a novel role in pigmentation. Development 137, 4083–4090

Zou FM, Lee KS, Kim BY, Kim HJ, Gui ZZ, Zhang GZ, *et al*. (2015) Differential and spatial regulation of the prophenoloxidase (proPO) and proPO-activating enzyme in cuticular melanization and innate immunity in *Bombyx mori* pupae. J Asia-Pac Entomol 18, 757–764.