

Serine Protease MP2 Activates Prophenoloxidase in the Melanization Immune Response of *Drosophila melanogaster*

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Abstract

In arthropods, melanization plays a major role in the innate immune response to encapsulate and kill the invasive organisms. It is mediated by a serine protease cascade and is regulated by serpins. The identification of the molecular components of melanization and the regulation of those components are still unclear in *Drosophila melanogaster*, although some genetic research on the activation of melanization has been reported. Here we report that *Drosophila* serine protease MP2 directly cleaves both recombinant and native prophenoloxidase-1. Overexpression or repression of MP2 in flies resulted in increased and decreased rates of cleavage, respectively, of prophenoloxidase-1. Moreover, serine protease inhibitor Spn27A formed SDS-stable complexes with MP2, both *in vitro* and *in vivo*. The amidase activity of MP2 was inhibited efficiently by Spn27A. Spn27A also prevented MP2 from cleaving prophenoloxidase-1. Taken together, these results indicate that under our experimental conditions MP2 functions as a prophenoloxidase-activating protease, and that this function is inhibited by Spn27A. MP2 and Spn27A thus constitute a regulatory unit in the prophenoloxidase activation cascade in *Drosophila*. The combination of genetic, molecular genetic and biochemical approaches should allow further advances in our understanding of the prophenoloxidase-activating cascade in insects and indirectly shed further light on protease-cascades in humans and other vertebrates.

Citation: An C, Zhang M, Chu Y, Zhao Z (2013) Serine Protease MP2 Activates Prophenoloxidase in the Melanization Immune Response of *Drosophila melanogaster*. PLoS ONE 8(11): e79533. doi:10.1371/journal.pone.0079533

Editor: François Leulier, Ecole Normale Supérieure de Lyon, France

Received: July 31, 2013; **Accepted:** September 29, 2013; **Published:** November 15, 2013

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Funding: This research was supported by National Natural Science Foundation of China (31172090), Program for New Century Excellent Talents in University (NCET-11-0476), and the 973 Program (2013CB114102). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Insects rely on their innate immune system to defend against invasion by pathogens or parasites [1]. Genetic and molecular approaches have revealed striking similarities between the mechanisms that regulate insect host defense and the mammalian innate immune response. In insect innate immune response, besides the well-documented induction of antimicrobial peptides through the Toll and Imd pathways [2–4], melanization functions to encapsulate and kill invading microbes, and also cooperates with other immune responses such as blood coagulation, wound healing, phagocytosis, and antimicrobial peptide expression [5,6]. Fruit fly, *Drosophila melanogaster*, has extensively been used to study molecular mechanisms involved in the activation and regulation of innate immune responses. However, our knowledge about the melanization response in *Drosophila* is rather limited compared to the characterization of the Toll signaling pathway. Studies on the mechanism of melanization have focused more on relatively large insects such as the silkworm, *Bombyx mori* [7–9], the tobacco hornworm, *Manduca sexta* [10–12], and the beetle *Tenebrio molitor* [13,14], none of which have the wealth of genetic techniques that are available in *Drosophila*.

During melanization, phenoloxidase (PO) catalyzes hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-

diphenols to quinones, which then polymerize to form melanin [15]. Melanin and cytotoxic molecules produced in this process, including quinones and reactive oxygen intermediates, may sequester and kill the intruding microorganisms [16]. Arthropod POs are produced as inactive zymogens called prophenoloxidase (PPO), and the activation of PPO is one of the rate-limiting steps in melanization [5]. PPO activation is mediated by a serine protease cascade, which is somewhat analogous to the coagulation pathway and complement system in human plasma [17,18]. Biochemical studies in *M. sexta* and *T. molitor* have led to the current model of PPO activation [10,11–14,19]. Soluble pattern-recognition proteins initially recognize non-self molecular patterns in invading organisms or from aberrant host tissues. This interaction triggers activation of a series of serine proteases, culminating in the activation of prophenoloxidase-activating protease (PAP), also known as PPO-activating enzyme or factor (PPAE or PPAF) [10,11–14]. Activated PAP converts inactive PPO to PO by cleaving PPO at an Arg-Phe bond at approximately residue 50 [12,18,20–24]. PAPs have been identified from several arthropods, including *B. mori* [7], *M. sexta* [12,18,20], the mosquito, *Anopheles gambiae* [25], two beetles, *Holotrichia diomphalia* and *T. molitor* [14,22], and a crayfish, *Pacifastacus leniusculus* [23]. All of these PAPs belong to a family of serine proteases containing a clip

domain (a family therefore called clip-domain serine proteases). The proteases have a C-terminal catalytic domain and one or two N-terminal clip domains, connected by a disulfide bond [26]. Genetic evidence in *Drosophila* indicates that three clip-domain proteases MP1 (CG1102), MP2 (CG3066), and Hayan (CG6361) are involved in melanization [27,28,29]. Hayan directly converts PPO to PO in systemic wound response [29]. Whether MP1 or MP2 directly activates *Drosophila* PPO is unknown.

PPO activation is highly regulated, presumably because cytotoxic intermediates generated by uncontrolled PPO activation would be harmful to the insect [30]. Serine protease cascades that contribute to PPO activation are often controlled by members of the serine protease inhibitor (serpin) superfamily [31,32]. Serpins contain ~400 amino acid residues with an exposed reactive-center loop near their carboxyl terminus [33]. They function as suicide-substrate inhibitors by forming irreversible complexes with target proteases after the cleavage of a scissile bond (designated P1–P1') in the reactive-center loop [33–35]. A group of orthologous serpins which negatively regulate PAPs has been identified in several insects, including *An. gambiae* SRPN2 [25], *M. sexta* serpin-3 [34], *T. molitor* Spn48 [35]. Six serpins, including Spn27A, Spn28D, Spn43Ac, Spn77Ba, Spn4, and Spn5, have been studied functionally in *Drosophila* [36–42]. Spn27A inhibits Easter in the establishment of embryonic dorsoventral polarity, and also controls an unknown protease in melanization [36,43,44]. Recombinant Spn27A inhibits *H. diomphalia* PPAE *in vitro* [36], but has no direct regulation on Hayan [29]. The endogenous targets of Spn27A in *Drosophila* are still unknown.

Our current knowledge about melanization in *Drosophila* is mainly obtained from genetic studies. Here we report methods to activate purified recombinant MP2. We then use this purified enzyme in biochemical assays - in combination with genetic techniques - to investigate the molecular mechanisms of *Drosophila* PPO activation. Our results indicate that MP2 directly cleaves and activates *Drosophila* prophenoloxidase 1 (PPO1) and, therefore, functions as a PAP in *Drosophila*. Moreover, Spn27A is found to inhibit MP2 activity. MP2 and Spn27A thus constitute a regulatory unit in the PPO activation cascade in *Drosophila*.

Results

Phylogenetic Analysis of *Drosophila* Serine Proteases

Serine proteases containing clip domains are implicated in PPO activation in arthropods [26]. To identify putative candidate proteases that might function as PAPs in *Drosophila*, we initially checked the sequences of 47 clip-domain serine proteases and serine protease homologs retrieved from the ImmunoDB. Among those, only 26 proteins possessed clip domain as well as a catalytic triad with the characteristics of active serine proteases. A phylogenetic analysis was then performed based on the deduced amino acid sequences of these 26 selected *Drosophila* sequences and others known to be involved in activation of melanization reaction in other species. As shown in Fig. S1 and Fig. S2, many of the clades in the phylogeny have low support. One clade, with a bootstrap value of 34, includes *Drosophila* MP2 (CG3066), *An. gambiae* CLIPB9 and *H. diomphalia* PPAF1. Another clade, with a bootstrap value of 52, groups *Drosophila* CG9737 with *M. sexta* PAP-2 and -3, and with *B. mori* PPAE. All of the proteases that cluster with *Drosophila* MP2 or CG9737 function as a PAP in initiating melanization [12,18,20,25]. MP2 was reported to participate in melanization process in *Drosophila* [27,28]. However, its substrate is still unknown and therefore we focused on it in the work reported here.

MP2 Sequence Analysis

Drosophila MP2 consists of 391 amino acid residues including a predicted 27-residue secretion signal peptide. The calculated mass and isoelectric point of mature proMP2 are 41,260 Da and 6.11. There are nine potential O-linked glycosylation sites and one potential N-linked glycosylation site (Fig. S3). MP2 is composed of an amino-terminal clip domain connected by a linker region to a carboxyl-terminal catalytic domain containing three conserved amino acid residues: histidine, aspartic acid, and serine [45]. The predicted proteolytic activation site is located at FSNK¹³⁶↓VYNG (Fig. S3). After cleavage, the resulting polypeptide fragments containing the clip domain and the catalytic domain would be expected to remain connected by an interchain disulfide bond (Cys¹²⁸–Cys²⁶⁴).

Production of Recombinant proMP2, proMP2_{Xa}, Spn27A, and DmPPO1

In order to investigate the roles of MP2 and Spn27A in *Drosophila*, we expressed and purified them as recombinant proteins. MP2 was expressed in zymogen form. To obtain the activated form of MP2, we mutated its predicted activation site to allow its activation by bovine Factor Xa. This mutant was named as proMP2_{Xa}. We have used this approach previously to prepare zymogens that can be activated by the commercially available Factor Xa [10,25]. Both recombinant proMP2 and proMP2_{Xa} were secreted from Sf9 cells using the MP2 secretion signal peptide. SDS-PAGE analysis indicated that proMP2 and proMP2_{Xa} had a similar apparent molecular mass of 49 kDa (Fig. 1), slightly larger than that predicted from the cDNA sequences (41.3 kDa). The increased mass is likely due to glycosylation, as the protein sequence contains ten potential glycosylation sites (Fig. S3). Spn27A and PPO1 were expressed in *E. coli*. SDS-PAGE analysis demonstrated that purified Spn27A and PPO1 had a mass of ~48 and ~79 kDa, respectively, similar to predicted mass based on their sequences (Fig. 1).

Activation of Recombinant proMP2_{Xa} by Factor Xa

To detect activation of proMP2_{Xa} by Factor Xa, we performed immunoblot analysis and amidase activity assays (Fig. 2). Incuba-

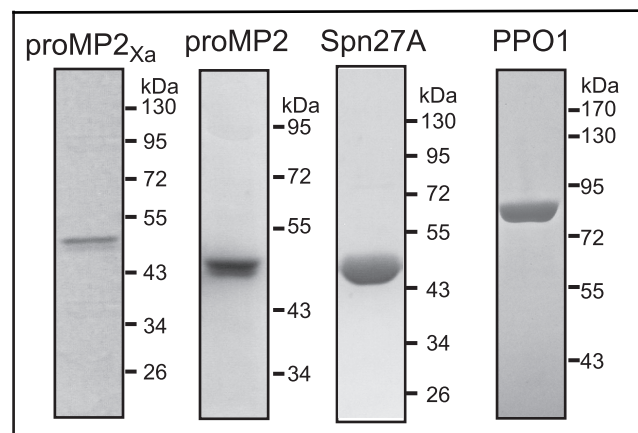


Figure 1. SDS-PAGE analysis of recombinant proMP2_{Xa}, proMP2, Spn27A and PPO1. The purified recombinant proMP2_{Xa} (0.7 μg), proMP2 (0.99 μg) and Spn27A (2.6 μg) were treated with SDS sample buffer containing β-mercaptoethanol and separated by 10% SDS-PAGE followed by coomassie brilliant blue staining. Recombinant PPO1 (1.3 μg) was separated by 7.5% SDS-PAGE. The sizes and positions of the molecular mass standards are indicated on the right. doi:10.1371/journal.pone.0079533.g001

tion of purified proMP2_{Xa} with Factor Xa resulted in decreased intensity of the 49-kDa zymogen band and appearance of a 36-kDa band corresponding to the catalytic domain (Fig. 2A), as expected for activation. This result indicates that Factor Xa effectively cleaved proMP2_{Xa}. In addition to the 36-kDa band, a band with apparent molecular weight of ~30 kDa was detected by anti-His antibodies. This 30-kDa band might be due to the non-expected cleavage of proMP2_{Xa} by Factor Xa, as this band also appeared in the reaction mixture of Factor Xa and proMP2, which lacks IEGR sites.

To confirm the activation of proMP2_{Xa} by Factor Xa, we assayed for amidase activity of MP2_{Xa} using the colorimetric substrate IEARpNA. A significant increase in activity (above that of Factor Xa alone) was observed in the presence of MP2_{Xa} that was activated by Factor Xa (Fig. 2B), thus confirming activation of proMP2_{Xa}.

MP2_{Xa} Directly Cleaves and Activates *Drosophila* PPO1 in Hemolymph and *in vitro*

To characterize the role of MP2 in *Drosophila* PPO activation cascade, we incubated Factor Xa-activated MP2_{Xa} with hemolymph from *w¹¹¹⁸* control flies. Immunoblot analysis, using *Drosophila* anti-PPO1 antibodies, identified a single band with the molecular weight of ~90 kDa in hemolymph alone, which represented native *Drosophila* PPO1. No change was observed after incubation the hemolymph with Factor Xa or proMP2_{Xa} zymogen (Fig. 3A, left panel). However, when Factor Xa-activated MP2_{Xa} was incubated with the hemolymph, a band of ~83 kDa, corresponding to active phenoloxidase-1, was detected (Fig. 3A, left panel), indicating that active MP2_{Xa} causes predicted cleavage of *Drosophila* PPO1.

We incubated active MP2_{Xa} with purified recombinant *Drosophila* PPO1 to explore the cleavage of this zymogen by MP2 *in vitro*. Antibodies against *Drosophila* PPO1 detected this recombinant protein as a main band with the apparent molecular weight of ~90 kDa (Fig. 3A, right panel). No change was observed in the mixtures containing recombinant PPO1 and proMP2_{Xa}

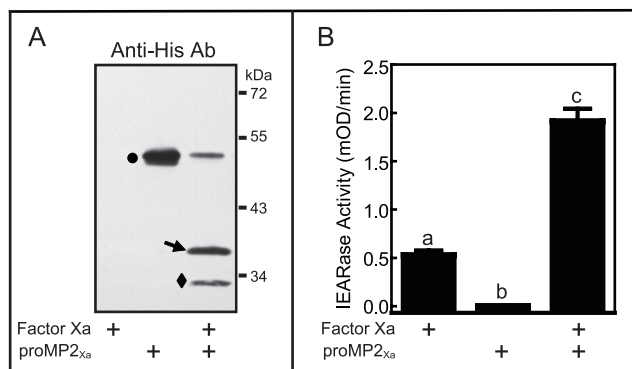


Figure 2. Activation of purified proMP2_{Xa} by bovine Factor Xa. (A) Purified recombinant proMP2_{Xa} (350 ng) was incubated with Factor Xa (200 ng) at 37°C for 1 h, and the mixtures were separated by 10% SDS-PAGE followed by immunoblot analysis using Anti-His antibodies (diluted 1:1000). The sizes and positions of molecular weight standards are indicated on the right. Circle, arrow, and diamond indicated proMP2_{Xa}, catalytic domain of proMP2_{Xa}, and non-specific cleavage of proMP2_{Xa}, respectively. (B) Amidase activity assay of activated MP2_{Xa} using IEARpNA as a substrate, as described under "Materials and methods". The bars represent mean ± S.D. (n=3). Bars labeled with different letters (a, b, and c) are significantly different (analysis of one-way ANOVA followed by Newman-Keuls test, P<0.05). doi:10.1371/journal.pone.0079533.g002

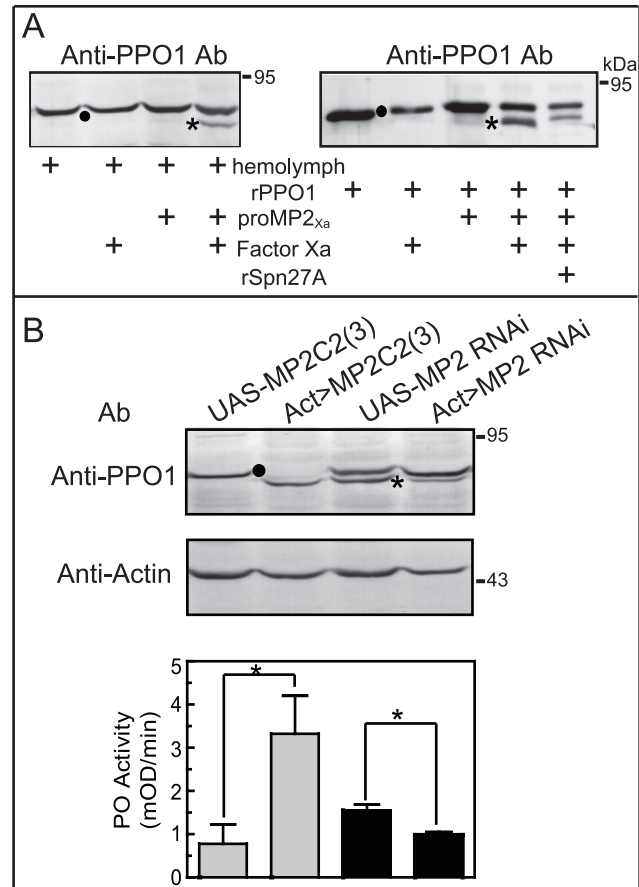


Figure 3. MP2 directly cleaves *Drosophila* PPO1. (A) MP2_{Xa} cleaves *Drosophila* PPO1 in hemolymph (Left) or as purified proteins (Right). Asterisks cleaved and activated PPO1; circle, PPO1 zymogen. (B) MP2 cleaves native PPO1 in transgenic flies. Hemolymph was collected from flies with the overexpression of preactivated MP2 (*Act>MP2C2*) or depletion of MP2 (*Act-MP2RNAi*), and respective control flies. Samples were subjected to immunoblot analysis using Anti-PPO1 antibodies (upper panel) and PO activity assay (lower panel). The bars represent mean ± S.D. (n=3). Asterisks indicate means that are significantly different from control (unpaired t test, P<0.05). doi:10.1371/journal.pone.0079533.g003

zymogen but incubation of the recombinant PPO1 with Factor Xa-activated MP2_{Xa} resulted in decreased intensity of the 90 kDa zymogen and the appearance of an immunoreactive band at ~83 kDa, the expected size for activated phenoloxidase-1 (Fig. 3A, right panel). Pre-incubation of Spn27A and MP2_{Xa} diminished the intensity of this band, suggesting that Spn27A prevented MP2 from cleaving *Drosophila* PPO1.

To further investigate the roles of MP2 in melanization in flies, hemolymph from flies with either overexpression (*Act>MP2C2*) or repression (*Act>MP2 RNAi*) of MP2 was collected for immunoblot analysis and phenoloxidase activity assay. Anti-PPO1 antibodies recognized a ~90-kDa band corresponding to PPO in control hemolymph (*UAS-MP2C2*). This band disappeared, and an immunoreactive band at ~83-kDa (the expected active band of phenoloxidase-1) was observed in hemolymph overexpressing pre-activated MP2 (*Act>MP2C2*) (Fig. 3B, upper panel). The phenoloxidase activity of this hemolymph increased significantly (Fig. 3B, lower panel). Conversely, the intensity of the ~83-kDa band decreased, compared to controls (*UAS-MP2 RNAi*), in hemolymph from flies lower expressing MP2 (Fig. 3B, upper

panel), and phenoloxidase activity decreased significantly (Fig. 3B, lower panel).

Spn27A Inhibits MP2 Activity

The formation of an SDS-stable complex of serpin with a protease it inhibits is a characteristic feature of a serpin-protease reaction [33]. As a first step to determine if MP2 can be directly inhibited by Spn27A, we tested whether these two proteins form such a complex *in vitro*. Purified recombinant Spn27A and Factor Xa-activated MP2_{Xa} were combined for SDS-PAGE and immunoblotting with anti-His or anti-Spn27A antibodies (Fig. 4A). Anti-His antibodies recognized the 49-kDa MP2_{Xa} zymogen and 48-kDa recombinant Spn27A, which could not be separated on 10% SDS-PAGE, and the 36-kDa active MP2_{Xa} (Fig. 4A, left panel). When Spn27A was combined with active MP2_{Xa}, the intensity of the 36-kDa band corresponding to the MP2 catalytic domain decreased and an immunoreactive band at ~80-kDa (the expected size of the Spn27A/MP2_{Xa} complex) appeared. This band was also recognized by anti-Spn27A antibodies (Fig. 4A, right panel). We also expressed another recombinant serpin, Spn77Ba, which was also reported to regulate melanization in *Drosophila* [39]. When Factor Xa-activated MP2_{Xa} was mixed with Spn77Ba, no band with apparent high molecular weight (~80-kDa) was detected by Anti-His antibodies (Fig. S4). These results suggest that the formation of covalent complexes between MP2 and Spn27A is specific and that Spn27A is an inhibitor of MP2.

To test if this complex can be formed *in vivo*, we combined *Drosophila* hemolymph and recombinant MP2_{Xa}. Since endogenous Spn27A in wild-type flies might be not abundant, we collected hemolymph from flies overexpressing Spn27A (*act>Spn27A*). Hemolymph collected from adult flies bearing UAS-Spn27A constructs were used as controls. Native Spn27A in the hemolymph was detected at the position of 48 kDa by anti-Spn27A antibodies. This 48-kDa band was faint in UAS-Spn27A control flies, but it became strong after Spn27A was overexpressed via actin driver. When Factor Xa-activated MP2_{Xa} was mixed with such hemolymph, a higher molecular weight band of ~80 kDa in addition to the 48-kDa Spn27A band was detected by anti-Spn27A antibodies (Fig. 4B). This 80-kDa band was at the same position as complexes formed between recombinant MP2_{Xa} and recombinant Spn27A. Anti-Spn27A antibodies failed to detect any band corresponding to the SDS-stable Spn27A-MP2 complex when active MP2_{Xa} was mixed with control hemolymph (*UAS-Spn27A*) without the overexpression of Spn27A (Fig. 4B).

To further investigate the inhibition of MP2_{Xa} by Spn27A, we tested Spn27A's ability to inhibit hydrolysis of a colorimetric peptide substrate by MP2_{Xa}. MP2_{Xa} activity decreased linearly as Spn27A concentration increased (Fig. 4C). The stoichiometry of inhibition was 3.5, indicating that under the experimental conditions Spn27A preferentially acts as an inhibitor rather than a substrate of MP2 [33].

Discussion

Proteolytic activation of PPO is a critical step in the host defense system against invading pathogens and parasites and, as such, has been extensively investigated in various insects and crustaceans for more than 40 years [46]. Even so, understanding of the PPO activation cascade is still incomplete. Especially in *Drosophila*, current knowledge of this cascade is still limited. Studies of the PPO activation cascade in several relatively large insects, in which biochemical studies were feasible, have established the basic outlines of this cascade as well as aspects of its control by serpins. Those pioneering studies have set the scene for continued studies

in other insects in which the initial biochemical work would have been very difficult. We suggest that *Drosophila* is especially appropriate for such continued studies, because of the wide range of genetic and molecular genetic tools available in that system. In combination with the expression of recombinant proteins for biochemical studies, these genetic and molecular genetic tools will allow deeper understanding of PPO activation cascade in *Drosophila*. Furthermore, although PPO activation cascade does not exist in mammals, it is analogous to other protease-cascades in humans, especially complement activation and blood coagulation. As regards the roles of serpins in regulations of protease-cascades, we can expect that insights from studies in *Drosophila* and other insects will be relevant to better understand the control of analogous cascades in mammals. Therefore, in this research we combined biochemical and genetic techniques and investigated the molecular mechanisms of PPO activation in *Drosophila*.

Sequence analysis demonstrated that there are 26 genes encoding clip-domain serine proteases in the *Drosophila* genome (Fig. S1, Fig. S2 and Ref [47]). Among them, in our phylogenetic tree MP2 is clustered with *An. gambiae* CLIPB9 and *H. diomphalia* PPAF1, which are both known to function in melanization (Fig. S1 and Ref [24,25]). MP2 has been suggested to be involved in PPO activation because overexpression of activated MP2 resulted in constitutive melanization in larvae, pupae, and adults in *Drosophila*, whereas lack of MP2 produced a failure to activate melanization upon microbial challenge [27,28]. Therefore, MP2 was a good candidate to function in PPO activation. We therefore performed, and report here, *in vitro* and *in vivo* experiments to determine whether MP2 directly activates PPO. The addition of active MP2 to *Drosophila* hemolymph did result in cleavage of PPO1, and active MP2 also directly cleaved and activated recombinant *Drosophila* PPO1 *in vitro* (Fig. 3A). When the expression of MP2 was elevated or knocked down using Gal4/UAS system, the cleavage of PPO1 and the level of PO activity in hemolymph increased or decreased accordingly (Fig. 3B). These results suggest that MP2 functions as the terminal protease in the PPO activation pathway.

It is notable that residual non-cleavage of PPO1 remained detectable and low phenoloxidase activity still existed in flies expressing MP2 dsRNA for transcript knockdown via RNAi (Fig. 3B). A possible reason could be incomplete knockdown of MP2, potentially allowing for residual MP2 activity in *Drosophila*. Quantitative RT-PCR analysis showed that the transcript levels in MP2 repressing flies were reduced to roughly 45% (Fig. S5). Another probable explanation is the existence of other PAPs in *Drosophila* which share this function with MP2. It is conceivable that several PAPs work cooperatively to activate melanization reaction in one insect. For example, three such enzymes (named PAP1, PAP2, and PAP3) can cleave and activate PPO in *M. sexta* [12,18,20], although they themselves are activated by different serine proteases [10,11,19]. In *Drosophila*, Hayan has been identified as a PAP [29]. In addition, we identified in the *Drosophila* genome MP2 is most similar to *M. sexta* PAP1, and CG9737 has the highest similarity to *M. sexta* PAP2 and PAP3 (Fig. S1) in 26 clip-containing serine proteases. We therefore hypothesize that *Drosophila* CG9737 may also act as a PAP, and that its zymogen is activated by a protease different from that one activating proMP2. Tang *et al* reported MP1 and MP2 defined a melanization cascade in *Drosophila*, and MP1 is required to activate the melanization in response to both bacterial and fungal infection while MP2 is mainly involved in the infection of fungi [28]. MP1 potentially acts as a PAP to activate the PPO zymogen [28]. Based on the results presented in this study, we hypothesize that there exist multiple PAPs in *Drosophila*, and MP1, MP2, Hayan, and CG9737 could well be involved in different branches and respond

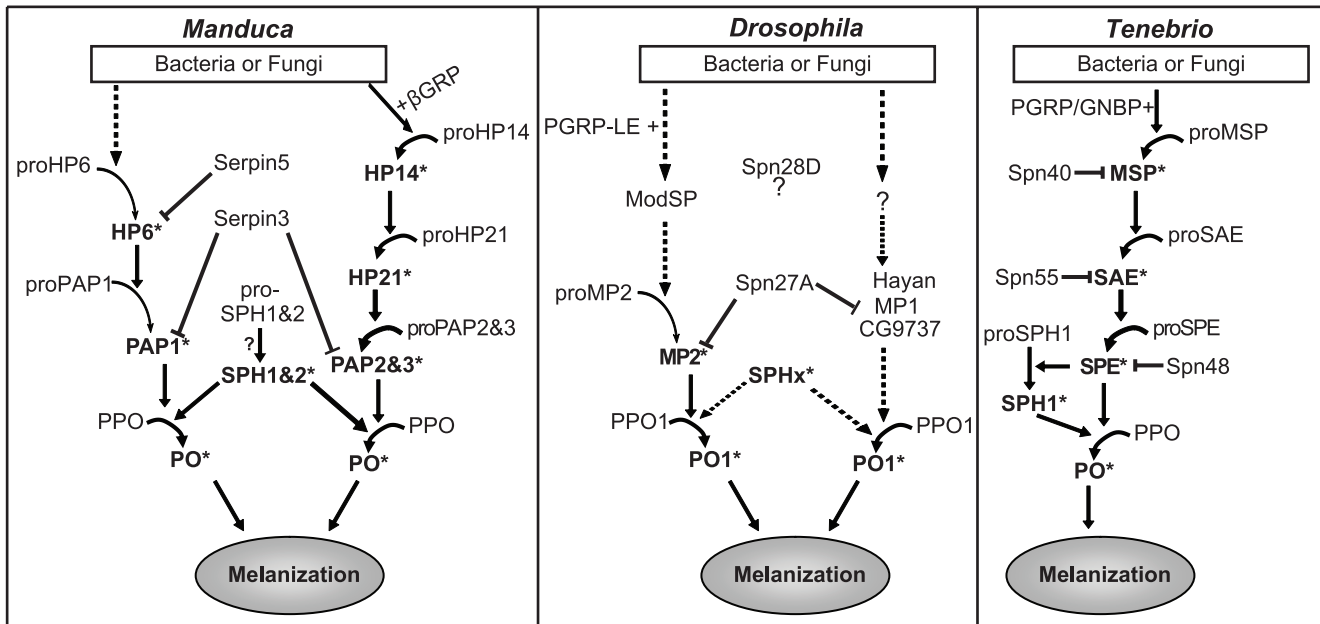


Figure 5. A model for activation of melanization in *Drosophila* and comparison with *M. sexta* and *T. molitor* melanization pathway [10–14,18,28,29,34,37,47,49,60,61]. Arrows indicate activation of downstream components or steps. Dashed arrows indicate steps that have not been experimentally verified. Regulation of proteases by serpins is indicated. doi:10.1371/journal.pone.0079533.g005

Spn27A; *MP2*^{KG02818} double-mutant flies presented spontaneous large melanization spots, with the same frequency as *Spn27A* single mutants [27]. They speculated that MP2 did not directly cleave PPO, but instead activated a downstream PAP which was also regulated by *Spn27A*. Based on the results from our experiments and the literature, we propose the following hypothesis to interpret the above observations: *Spn27A* inhibits MP2 and another PAP (or proteases). When MP2 is inactivated by insertion of the P element KG02818, another (redundant) PAP (or proteases) such as Hayan or MP1 compensates for the deficiency of MP2. Hayan is involved in *Spn27A*-controlled PO activity, but not inhibited directly by *Spn27A* [29]. However, it is highly possible that MP1 is the direct target protease of *Spn27A*, because induction of PO activity by overexpression of preactivated MP1 was blocked by simultaneous overexpression of *Spn27A* [28]. Experiments are underway to study the possible regulation of *Spn27A* on MP1.

In this study, we identified *Drosophila* MP2 as a PAP, and found that MP2 is inhibited by *Spn27A* in activating melanization. Based on our work reported here and other relevant observations as cited above, we propose a model for activation and regulation of melanization in *Drosophila*. Upon bacterial or fungal infection, specific patterns on the surface of invading pathogens are recognized by pattern recognition protein, such as PGRP-LE [50]. Then, an unknown, modular serine protease is activated in the presence of pattern recognition protein. Once activated, this unknown protease processes a series of clip-domain serine proteases, which leads to activation of proMP2. Active MP2 then directly cleaves PPO to produce active PO (Fig. 5). In another activation branch, Hayan or MP1 or CG9737 functions as a distinct PAP. *Spn27A* inhibits MP2 and other PAPs. *Spn28D* does not inhibit MP2, but confines PO availability by controlling its initial release [38].

In addition, we also note that the efficiency of cleavage of PPO1 by MP2 *in vitro* was relatively low, although it did occur (Fig. 3A). Perhaps a protein in addition to MP2 is required *in vivo* for PPO activation. In fact the required involvement of proteins other than

PAPs for PPO activation has been demonstrated in *Hyalophora cecropia*, *H. diomphalia*, *M. sexta*, and *T. molitor* [13,51–53]. Clip-domain serine protease homologs whose catalytic serines are replaced with glycine and therefore lack proteolytic activity are required for generating active PPO, enhancing the cleavage by PAPs. We therefore suggest that also in *Drosophila* serine-protease homologs function as “protein cofactors” to facilitate PPO activation. Bioinformatic analysis indicates that there are 19 genes for serine-protease homologs in *Drosophila* [47]. Experiments to examine their functions are underway in our laboratory.

Materials and Methods

Drosophila Stocks

Flies were maintained on standard cornmeal-yeast medium at 25°C under a relative humidity of 60% and a photoperiod of 12 h light. Fly strain *w*¹¹¹⁸ was used as the wild-type control unless otherwise specified. Other transgenic fly strains used were as follows: *act-Gal4*, *UAS-Spn27A* [36], *UAS-MP2C2(3)* [28], *UAS-MP2Ri1(3)* [28]. Transgenes were expressed using the Gal4-UAS system [54].

Phylogenetic Analysis of Serine Proteases in *Drosophila*

To identify serine proteases that are potentially involved in melanization in *Drosophila*, protein sequences of all annotated, putatively active serine proteases were retrieved from the *Drosophila* genome (<http://cegg.unige.ch/Insecta/immunodb>). Their catalytic-domain sequences were aligned with serine proteases from other species known to have a function related to melanization using the Clustal W program. Phylogenetic trees were constructed by the neighbor-joining method using MEGA version 4 software [55]. For the neighbor-joining method, gaps were treated as characters, and statistical analysis was performed by the bootstrap test, with 1,000 repetitions. The sequences (with GenBank accession number) used for the alignment with *Drosophila* serine proteases were: *B. mori* BAEase (ABB58762) and PPAE (NP_001036832);

H. diomphalia PPAF1 (BAA34642); *M. sexta* HP6 (AAV91004), HP8 (AAV91006), HP13 (AAV91011), HP15 (AAV91012), HP21 (AAV91019), PAP1 (AAX18636), PAP2 (AAL76085), and PAP3 (AAO74570); *T. molitor* SAE (AB363979) and SPE (AB363980).

Preparation of Recombinant proMP2, proMP2_{Xa}, Spn27A and PPO-1

To biochemically characterize roles and regulation of MP2 in *Drosophila* melanization, we expressed recombinant proMP2, Spn27A, and PPO-1 using baculovirus or prokaryote system. For the production of proMP2, primers (Table S1) designed based on the nucleotide sequences in FlyBase were used to amplify the full length of proMP2 from cDNA of adult flies. The PCR product was recovered and ligated to pMD19-T vector. The resulting plasmid was used as template to amplify the entire proMP2 coding region, including the signal peptide, using primers listed in Table S1. The forward primer included a *SpeI* site, and the reverse primer contained three codons for glycine and six codons for histidine residues followed by a stop codon and a *HindIII* site. The PCR product was recovered by agarose gel electrophoresis, digested with *SpeI* and *HindIII* and then inserted into the corresponding restriction sites in the vector pFastBacl (Invitrogen). The resulting proMP2 plasmid, after sequence confirmation, was used as template to produce mutant proMP2 (proMP2_{Xa}) plasmids following Chiu's methods [56]. In proMP2_{Xa}, residues 133–136 at the predicted activation site of proMP2 were changed from FSNK¹³⁶ to IEGR¹³⁶ to permit its activation by bovine Factor Xa [57]. After sequence verification, proMP2 and proMP2_{Xa} plasmids were used to generate a recombinant baculovirus using Cellfectin[®] II Reagent (Invitrogen). To express proMP2 and proMP2_{Xa}, Sf9 cells (2×10^6 cells/ml) in 500 ml of Insect-Xpress protein-free medium (Lonza) were infected with the recombinant baculovirus at multiplicity of infection of 5 and were incubated at 28°C with shaking at 150 rpm. The culture was harvested at 48 h post infection, and cells were removed by centrifugation at $5000 \times g$ for 20 min at 4°C. The cell-free medium was used to further purify recombinant proMP2 and proMP2_{Xa} following the method described previously [58].

To express Spn27A, a cDNA fragment encoding mature Spn27A was amplified by PCR using gene-specific primers (seen in Table S1). The forward primer contained an *NcoI* restriction site, which provided the start codon, followed by six codons for histidine residues. The reverse primer included an *XhoI* site at the 3' end of stop codon. The PCR product was cloned into pMD19 T-vector. The resulting plasmid was used as template to produce silent mutated plasmids with Chiu's methods [56]. In plasmids with silent mutation, nucleotides 965–970 were changed from CCATGG to CTATGG, which prevented digestion by *NcoI* but retained the original amino acid sequence. After sequence verification, the mutant plasmids were excised with *NcoI* and *XhoI* and subcloned into the same restriction sites in the expression vector pET-28a (Novagen). The resulting plasmid was used to transform *E. coli* BL21 (DE3) strain. For recombinant Spn27A expression, these bacteria were grown at 37°C in LB medium containing 50 µg/ml of kanamycin. When OD₆₀₀ of the culture reached 0.8, isopropyl β-D-thiogalactoside was added to a final concentration of 0.1 mM, and the recombinant protein was expressed for 10 h at 25°C. The bacteria were harvested by centrifugation at $4500 g$ for 20 min, and resuspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0). After being lysed by sonication, soluble Spn27A in the cleared lysate was purified by nickel-nitrilotriacetic acid (NTA) agarose chromatography as described by An *et al* [58].

For expressing recombinant *Drosophila* PPO-1, plasmids in which a fragment encoding full length of prophenoloxidase-1 was inserted into the restriction sites *NcoI* and *NdeI* in vector pET28b (Novagen) were kindly provided by Dr. Erjun Ling (Institute of Plant Physiology and Ecology, Chinese Academy of Sciences), and used to transform *E. coli* strain BL21(DE3). The protein was then expressed and purified following the methods described by Li *et al* [59].

SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblot Analysis

Protein concentrations were determined using Bradford Reagent Solution (Sangon) with bovine serum albumin as a standard. For SDS-PAGE, protein samples were treated with $5 \times$ SDS sample buffer containing β-mercaptoethanol at 95°C for 5 min and then separated by 10% SDS-PAGE. Proteins were detected by staining with coomassie brilliant blue. For immunoblot analysis, proteins were transferred onto a nitrocellulose membrane and detected with mouse anti-His (1:1000), or rabbit anti-*Drosophila* PPO1 (1:2,000, generously provided by Dr. Erjun Ling) as primary antibodies. Antibody binding was visualized using alkaline phosphate-conjugated goat anti-mouse or anti-rabbit IgG (diluted 1:2000) and 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT) staining buffer containing 165 µg/ml BCIP and 330 µg/ml NBT in 100 mM Tris (pH 9.5), 150 mM NaCl, and 5 mM MgCl₂.

Activation of Recombinant proMP2_{Xa} by Factor Xa

To test whether proMP2_{Xa} could be activated by Factor Xa, 350 ng of purified recombinant proMP2_{Xa} was incubated with 200 ng of bovine Factor Xa (New England Biolabs) in the reaction buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM CaCl₂, pH8.0) at 37°C for 1 h. The mixtures were separated by 10% SDS-PAGE followed by immunoblot analysis. The activation of proMP2_{Xa} was confirmed by measuring the amidase activity of activated MP2_{Xa} with 200 µl of 50 µM acetyl-Ile-Glu-Ala-Arg-*p*-nitroanilide (IEAR_pNA) in 0.1 M Tris-HCl (pH8.0), 0.1 M NaCl and 5 mM CaCl₂ as colorimetric substrate. The amidase activity was measured by monitoring A₄₀₅ in a microplate reader (Bio-Tek Instrument, Inc.). One unit of amidase activity was defined as $\Delta A_{405}/\text{min} = 0.001$.

PPO Activation Assays

To investigate the role of MP2 in activating *Drosophila* PPO-1 *in vitro*, 350 ng of proMP2_{Xa} or Factor-Xa activated MP2_{Xa} were incubated with 380 ng of purified recombinant PPO-1 or 3.5 µl of hemolymph from *w*¹¹¹⁸ adult flies at 37°C. After one hour, reaction mixtures were subjected to 7.5% SDS-PAGE and immunoblot analysis.

To measure the ability of MP2 to activate PPO-1 *in vivo*, hemolymph was collected from adult flies bearing the catalytic domain of MP2 or of MP2 RNAi constructs. Respective control flies were also used for hemolymph collection simultaneously. In order to collect the hemolymph from flies with MP2 RNAi constructs, adult flies were pierced with a needle of 0.1 mm diameter previously dipped into a concentrated *E. coli* culture [28]. Samples were subjected to SDS-PAGE and immunoblot analysis. Additionally, phenoloxidase activity in the collected hemolymph was measured using dopamine as substrate [10]. One unit of phenoloxidase activity was defined as the amount of enzyme producing an increase in absorbance (ΔA_{470}) of 0.001 per min.

Detection of SDS-stable Complexes Between MP2_{Xa} and Spn27A

To detect the formation of covalent complexes between MP2 and Spn27A *in vitro*, recombinant proMP2_{Xa} (350 ng) was activated by Factor Xa as described above and mixed with purified Spn27A at a molar ratio of 1:1. In control samples, proMP2_{Xa} or Factor Xa was omitted from the mixture. After incubation at room temperature for 30 min, the reaction mixtures were subjected to 10% SDS-PAGE and immunoblot analysis.

To detect formation of the MP2-Spn27A complex *in vivo*, hemolymph (insect blood) from 15 one-day old *UAS-Spn27A* or *act>Spn27A* adult flies was collected into phosphate-buffered saline as described [28], and mixed with 200 ng of Factor Xa-activated MP2_{Xa}. After incubation at 37°C for 30 min, samples were subjected to SDS-PAGE and immunoblot analysis.

Inhibition of MP2_{Xa} Activity by Spn27A

To assess the inhibitory potential of Spn27A towards MP2, proMP2_{Xa} (360 ng) was activated by Factor Xa and mixed with recombinant Spn27A at different molar ratios (0.5:1 to 10:1) in 30 µl of buffer (150 mM NaCl, 20 mM Tris, pH8.0). In control reactions, the same amount of Factor Xa used to activate proMP2_{Xa} was substituted for active MP2_{Xa}. After incubation at room temperature for 30 min, residual amidase activity was measured as described above. Amidase activity of MP2_{Xa} was defined as the activity of MP2_{Xa} minus the activity of Factor Xa alone.

Supporting Information

Figure S1 Phylogenetic analysis of the catalytic domains in clip-domain serine proteases from *Drosophila* and other insect species. The tree was derived from sequence alignment using Clustal W. Numbers at the nodes indicate the bootstrap confidence values of 1000 replicates. The scale bar indicates the number of substitutions per site. A very similar tree was obtained when the clip domains were included in the alignment. The circled bootstrap values indicate two clades that group *Drosophila* MP2 or CG9737 with other proteases involved in melanization reactions, respectively. Sequence identifiers for *Drosophila* proteases include Flybase number followed by a name given in the references if this protease has been studied functionally. The other sequences used for the analysis were: *Anopheles gambiae* CLIPB8, CLIPB9 and CLIPB10; *Bombyx mori* BAEEase and PPAE; *Holotrichia diomphalia* PPAF1; *Manduca sexta* HP6, HP8, HP13, HP15, HP21, PAP1, PAP2, and PAP3; *Tenebrio molitor* SAE and SPE.
(EPS)

Figure S2 Sequences used in alignment of the catalytic domains in clip-domain serine proteases from *Drosophila* and other insect species. Totally forty-two amino acid sequences were aligned for estimating the tree shown in FigureS1. Sequences were retrieved from ImmunoDB (<http://cegg.unige.ch/Insecta/immunodb>) and checked manually. Species acronym is defined as follows: *Anopheles gambiae*, Ag; *Bombyx mori*, Bm; *Holotrichia diomphalia*, Hd; *Manduca sexta*, Ms; *Tenebrio molitor*, Tm. *Drosophila* proteases are indicated by the corresponding Flybase numbers.
(PDF)

Figure S3 Sequence analysis of *Drosophila* MP2. The deduced amino acid sequences of MP2 are retrieved from

GenBank™. The predicted secretion signal peptide is double-underlined. The proteolytic activation site is indicated with “↓”. Three amino acid residues (Histidine, Aspartic acid, and Serine) critical for the catalytic activity of MP2 are shown in bold. Putative N-linked and O-linked glycosylation sites are shaded. Clip domain, linker region, and catalytic domain are shown. The absolutely conserved cysteines in clip domain are bold-italicized and numbered. The paired numbers (1–1, 2–2, 3–3) indicate the intramolecular disulfide linkage. Another two cysteines with numbers (4–4) indicate the disulfide linkage between clip domain and catalytic domain, which remains two domains connected after cleavage at the activation site. Nine amino acid residues (GGGHHHHHH) added to the carboxyl terminal for the expression of recombinant MP2 are italicized.
(EPS)

Figure S4 No complex formed between MP2_{Xa} and Spn77Ba. ProMP2_{Xa} (350 ng) was activated by Factor Xa and then incubated with Spn77Ba (450 ng) for 30 min at room temperature. In control reactions, proMP2_{Xa} or Factor Xa was omitted. The samples were subjected to 10% SDS-PAGE and immunoblot analysis using anti-His antibodies (diluted 1:1000). Size and positions of molecular mass standard are indicated to the right of the blot. No band with high molecular weight, consistent with the expected size for a serpin-protease complex, was recognized by anti-His antibodies. It suggests that MP2_{Xa} has no potential to form SDS-stable complex with Spn77Ba.
(EPS)

Figure S5 MP2 knockdown efficiency in MP2 repressing flies. Expression levels were measured by quantitative RT-PCR, using ribosomal protein S49 mRNA levels for normalization and UAS-MP2 RNAi samples as the calibrator.
(EPS)

Figure S6 Detection of cleavage of PPO1 (A) and PO activity (B) in flies over-expressing Spn27A. Transgenic flies were generated by combining one copy of a UAS-*Spn27A* construct with one copy of *act-Gal4* driver. Hemolymph was collected from *Act-Spn27A* transgenic flies and UAS-*Spn27A* control flies, and then subjected to immunoblot analysis using Anti-PPO1 antibodies (A) and PO activity (B). Circle, PPO1 zymogen; arrow, cleaved and activated PPO. The bars represent mean ± S.D. (n = 3). Asterisks indicate means that are significantly different from control (unpaired *t* test, *P*<0.05).
(EPS)

Table S1 Primer sequences.
(EPS)

Acknowledgments

We thank Dr. Yuhong Li for *act5-Gal4* fly stocks, Dr. Bruno Lemaitre for UAS-*Spn27A* fly stocks and antibodies against Spn27A; Dr. Carl Hashimoto for UAS-MP2C2(3) and UAS-MP2Ri1(3) fly stocks. We thank Dr. Erjun Ling for recombinant plasmids expressing PPO1 and antibodies against *Drosophila* PPO1. We are grateful to Dr. Michael R. Kanost and Gerald Reeck for suggestions on the manuscript.

Author Contributions

Conceived and designed the experiments: CA ZZ. Performed the experiments: CA MZ YC. Analyzed the data: CA MZ ZZ. Contributed reagents/materials/analysis tools: CA MZ YC. Wrote the paper: CA MZ YC ZZ.

References

- Iwanaga S, Lee BL (2005) Recent advances in the innate immunity of invertebrate animals. *J Biochem Mol Biol* 38: 128–150.
- De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B (2002) The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. *EMBO J* 21: 2568.
- Kambris Z, Brun S, Jang IH, Nam HJ, Romeo Y, et al. (2006) *Drosophila* immunity: a large-scale in vivo RNAi screen identifies five serine proteases required for Toll activation. *Curr Biol* 16: 808.
- Ferrandon D, Imler JL, Hetru C, Hoffmann JA (2007) The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections. *Nat Rev Immunol* 7: 862.
- Cerenius L, Lee BL, Soderhall K (2008) The proPO-system: pros and cons for its role in invertebrate immunity. *Trends Immunol* 29: 263.
- Kanost MR, Gorman MJ (2008) Phenoloxidases in insect immunity. In: Beckage NE, editor. *Insect Immunology*. Manhattan: Elsevier Academic Press. 69–96.
- Satoh D, Horii A, Ochiai M, Ashida M (1999) Prophenoloxidase-activating enzyme of the silkworm, *Bombyx mori*. Purification, characterization, and cDNA cloning. *J Biol Chem* 274: 7441.
- Clark KD, Strand MR (2013) Hemolymph melanization in the silkworm *Bombyx mori* involves formation of a high molecular mass complex that metabolizes tyrosine. *J Biol Chem* 288: 14476–14487.
- Shao Q, Yang B, Xu Q, Li X, Lu Z, et al. (2012) Hindgut innate immunity and regulation of fecal microbiota through melanization in insects. *J Biol Chem* 287: 14270–14279.
- An C, Ishibashi J, Ragan EJ, Jiang H, Kanost MR (2009) Functions of *Manduca sexta* hemolymph proteinases HP6 and HP8 in two innate immune pathways. *J Biol Chem* 284: 19716–19726.
- Gorman MJ, Wang Y, Jiang H, Kanost MR (2007) *Manduca sexta* hemolymph proteinase 21 activates prophenoloxidase-activating proteinase 3 in an insect innate immune response proteinase cascade. *J Biol Chem* 282: 11742–11749.
- Jiang H, Wang Y, Yu XQ, Kanost MR (2003) Prophenoloxidase-activating proteinase-2 from hemolymph of *Manduca sexta*. A bacteria-inducible serine proteinase containing two clip domains. *J Biol Chem* 278: 3552.
- Kan H, Kim CH, Kwon HM, Park JW, Roh KB, et al. (2008) Molecular control of phenoloxidase-induced melanin synthesis in an insect. *J Biol Chem* 283: 25316.
- Kim CH, Kim SJ, Kan H, Kwon HM, Roh KB, et al. (2008) A three-step proteolytic cascade mediates the activation of the peptidoglycan-induced toll pathway in an insect. *J Biol Chem* 283: 7599–7607.
- Nappi A, Poirie M, Carton Y (2009) The role of melanization and cytotoxic by-products in the cellular immune responses of *Drosophila* against parasitic wasps. *Adv Parasitol* 70: 99–121.
- Christensen BM, Li J, Chen CC, Nappi AJ (2005) Melanization immune responses in mosquito vectors. *Trends Parasitol* 21: 192–199.
- Kanost MR, Jiang H, Wang Y, Yu XQ, Ma C, et al. (2001) Hemolymph proteinases in immune responses of *Manduca sexta*. *Adv Exp Med Biol* 484: 319–328.
- Jiang H, Wang Y, Yu XQ, Zhu Y, Kanost M (2003) Prophenoloxidase-activating proteinase-3 (PAP-3) from *Manduca sexta* hemolymph: a clip-domain serine proteinase regulated by serpin-1J and serine proteinase homologs. *Insect Biochem Mol Biol* 33: 1049.
- Wang Y, Jiang H (2007) Reconstitution of a branch of the *Manduca sexta* prophenoloxidase activation cascade in vitro: snake-like hemolymph proteinase 21 (HP21) cleaved by HP14 activates prophenoloxidase-activating proteinase-2 precursor. *Insect Biochem Mol Biol* 37: 1015–1025.
- Jiang H, Wang Y, Kanost MR (1998) Pro-phenol oxidase activating proteinase from an insect, *Manduca sexta*: a bacteria-inducible protein similar to *Drosophila* easter. *Proc Natl Acad Sci* 95: 12220.
- Lee SY, Cho MY, Hyun JH, Lee KM, Homma KI, et al. (1998) Molecular cloning of cDNA for pro-phenol-oxidase-activating factor I, a serine protease induced by lipopolysaccharide or 1,3-beta-glucan in coleopteran insect, *Holotrichia diomphalia* larvae. *FEBS J* 257: 615.
- Lee SY, Kwon TH, Hyun JH, Choi JS, Kawabata SI, et al. (1998) *In vitro* activation of prophenoloxidase by two kinds of prophenoloxidase-activating factors isolated from hemolymph of coleopteran, *Holotrichia diomphalia* larvae. *FEBS J* 254: 50.
- Wang R, Lee SY, Cerenius L, Söderhall K (2001) Properties of the prophenoloxidase activating enzyme of the freshwater crayfish, *Pacifastacus leniusculus*. *FEBS J* 268: 895–902.
- Zou Z, Shin SW, Alvarez KS, Kokoza V, Raikhel AS (2010) Distinct melanization pathways in the mosquito *Aedes aegypti*. *Immunity* 32: 41.
- An C, Budd A, Kanost MR, Michel K (2011) Characterization of a regulatory unit that controls melanization and affects longevity of mosquitoes. *Cell Mol Life Sci* 68: 1929–1939.
- Jiang H, Kanost MR (2000) The clip-domain family of serine proteinases in arthropods. *Insect Biochem Mol Biol* 30: 95–105.
- Leclerc V, Pelte N, El Chamy L, Martinelli C, Ligoxygakis P, et al. (2006) Prophenoloxidase activation is not required for survival to microbial infections in *Drosophila*. *EMBO Rep* 7: 231–235.
- Tang H, Kambris Z, Lemaitre B, Hashimoto C (2006) Two proteases defining a melanization cascade in the immune system of *Drosophila*. *J Biol Chem* 281: 28097.
- Nam H, Jang I, You H, Lee K, Lee W (2012) Genetic evidence of a redox-dependent systemic wound response via Hyan protease-phenoloxidase system in *Drosophila*. *EMBO J* 31, 1253.
- Nappi AJ, Ottaviani E (2000) Cytotoxicity and cytotoxic molecules in invertebrates. *Bio Essays* 22: 469.
- Kanost MR (1999) Serine proteinase inhibitors in arthropod immunity. *Dev Comp Immunol* 23: 291.
- Silverman GA, Whisstock JC, Bottomley SP, Huntington JA, Kaiserman D, et al. (2010) Serpins flex their muscle: I. Putting the clamps on proteolysis in diverse biological systems. *J Biol Chem* 285: 24299–24305.
- Gettins PG (2002) Serpin structure, mechanism, and function. *Chem Rev* 102: 4751.
- Zhu Y, Wang Y, Gorman MJ, Jiang H, Kanost MR (2003) *Manduca sexta* serpin-3 regulates prophenoloxidase activation in response to infection by inhibiting prophenoloxidase-activating proteinases. *J Biol Chem* 278: 46556.
- Jiang R, Kim EH, Gong JH, Kwon HM, Kim CH, et al. (2009) Three pairs of protease-serpin complexes cooperatively regulate the insect innate immune responses. *J Biol Chem* 284: 35652.
- De Gregorio E, Han SJ, Lee WJ, Back MJ, Osaki T, et al. (2002) An immune-responsive serpin regulates the melanization cascade in *Drosophila*. *Dev Cell* 3: 581.
- Ligoxygakis P, Pelte N, Ji C, Leclerc V, Duvic B, et al. (2002) A serpin mutant links Toll activation to melanization in the host defence of *Drosophila*. *EMBO J* 21: 6330.
- Scherfer C, Tang H, Kambris Z, Lhocine N, Hashimoto C, et al. (2008) *Drosophila* Serpin-28D regulates hemolymph phenoloxidase activity and adult pigmentation. *Dev Bio* 323: 189.
- Levashina EA, Langley E, Green C, Gubb D, Ashburner M, et al. (1999) Constitutive activation of toll-mediated antifungal defense in serpin-deficient *Drosophila*. *Science* 285: 1917.
- Tang H, Kambris Z, Lemaitre B, Hashimoto C (2008) A serpin that regulates immune melanization in the respiratory system of *Drosophila*. *Dev cell* 15: 617.
- Richer MJ, Keays CA, Waterhouse J, Minhas J, Hashimoto C, et al. (2004) The Spn4 gene of *Drosophila* encodes a potent furin-directed secretory pathway serpin. *Proc Natl Acad Sci* 101: 10560.
- Ahmad ST, Sweeney ST, Lee JA, Sweeney NT, Gao FB (2009) Genetic screen identifies serpin5 as a regulator of the toll pathway and CHMP2B toxicity associated with frontotemporal dementia. *Proc Natl Acad Sci* 106: 12168.
- Hashimoto C, Kim DR, Weiss LA, Miller JW, Morisato D (2003) Spatial regulation of developmental signaling by a serpin. *Dev cell* 5: 945.
- Ligoxygakis P, Roth S, Reichhart JM (2003) A serpin regulates dorsal-ventral axis formation in the *Drosophila* embryo. *Curr Biol* 13: 2097.
- Jiang H, Wang Y, Gu Y, Guo X, Zou Z, et al. (2005) Molecular identification of a bevy of serine proteinases in *Manduca sexta* hemolymph. *Insect Biochem Mol Biol* 35: 931–943.
- Ashida M, Brey PT (1998) *Molecular Mechanisms of Immune Response in Insects*. London: Chapman & Hall. 135p.
- Ross J, Jiang H, Kanost MR, Wang Y (2003) Serine proteases and their homologs in the *Drosophila melanogaster* genome: an initial analysis of sequence conservation and phylogenetic relationships. *Gene* 304: 117.
- An C, Ragan EJ, Kanost MR (2011) Serpin-1 splicing isoform J inhibits the proSpätzle-activating proteinase HP8 to regulate expression of antimicrobial hemolymph proteins in *Manduca sexta*. *Dev Comp Immunol* 35: 135–141.
- Pike RN, Buckle AM, le Bonniec BF, Church FC (2005) Control of the coagulation system by serpins. Getting by with a little help from glycosaminoglycans. *FEBS J* 272: 4842–4851.
- Takehana A, Yano T, Mita S, Kotani A, Oshima Y, et al. (2004) Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in *Drosophila* immunity. *EMBO J* 23: 4690–4700.
- Lee KY, Zhang R, Kim MS, Park JW, Park HY, et al. (2002) A zymogen form of masquerade-like serine proteinase homologue is cleaved during pro-phenoloxidase activation by Ca²⁺ in coleopteran and *Tenebrio molitor* larvae. *FEBS J* 269: 4375–4383.
- Kim MS, Back MJ, Lee MH, Park JW, Lee SY, et al. (2002) A new easter-type serine protease cleaves a masquerade-like protein during prophenoloxidase activation in *Holotrichia diomphalia* larvae. *J Biol Chem* 277: 39999–40004.
- 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 Biochem Mol Biol* 33: 197.
- Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118: 401–415.
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 24: 1596–1599.
- Chiu J, March PE, Lee R, Tillett D (2004) Site-directed, Ligase-Independent Mutagenesis (SLIM): a single-tube methodology approaching 100% efficiency in 4 h. *Nucleic Acid Res* 32: e174.

57. Jenny RJ, Mann KG, Lundblad RL (2003) A critical review of the methods for cleavage of fusion proteins with thrombin and factor Xa. *Protein Express Purif* 31: 1.
58. An C, Jiang H, Kanost MR (2010) Proteolytic activation and function of the cytokine Spatzle in the innate immune response of a lepidopteran insect, *Manduca sexta*. *FEBS J* 277: 148.
59. Li X, Ma M, Liu F, Chen Y, Lu A, et al. (2012) Properties of *Drosophila melanogaster* prophenoloxidases expressed in *Escherichia coli*. *Dev Comp Immunol* 36: 648–656.
60. An C, Kanost MR (2010) *Manduca sexta* serpin-5 regulates prophenoloxidase activation and the Toll signaling pathway by inhibiting hemolymph proteinase HP6. *Insect Biochem Mol Biol* 40: 683–689.
61. Wang Y, Jiang H (2006) Interaction of beta-1,3-glucan with its recognition protein activates hemolymph proteinase 14, an initiation enzyme of the prophenoloxidase activation system in *Manduca sexta*. *J Biol Chem* 281: 9271.