Three Pairs of Protease-Serin Complexes Cooperatively Regulate the Insect Innate Immune Responses

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Serpins are known to be necessary for the regulation of several serine protease cascades. However, the mechanisms of how serpins regulate the innate immune responses of invertebrates are not well understood due to the uncertainty of the identity of the serine proteases targeted by the serpins. We recently reported the molecular activation mechanisms of three serine protease-mediated Toll and melanin synthesis cascades in a large beetle, Tenebrio molitor. Here, we purified three novel serpins (SPN40, SPN55, and SPN48) from the hemolymph of T. molitor. These serpins made specific serpin-serine protease pairs with three Toll cascade-activating serine proteases, such as modular serine protease, Spätzle-processing enzyme, and the specificity of the serine protease enzyme-activating enzyme, and Spätzle-processing enzyme and Spaetzle-processing enzyme-activating enzyme (SPE) (10). SPE has been identified as a terminal SP that cleaves pro-Spaetzle. Additionally, we provided biochemical evidence of the mechanism by which the GNBP3-mediated β-1,3-glucan recognition signal is transferred to pro-Spaetzle, leading to the production of AMPs (11). Furthermore, the terminally active serpins function as inducible negative feedback inhibitors. Unexpectedly, SPN55 and SPN48 were cleaved at Tyr and Glu residues in reactive center loops, respectively, despite being targeted by trypsin-like Spätzle-processing enzyme-activating enzyme and Spätzle-processing enzyme. These cleavage patterns are also highly similar to those of unusual mammalian serpins involved in blood coagulation and blood pressure regulation, and they may contribute to highly specific and timely inactivation of detrimental serine proteases during innate immune responses. Taken together, these results demonstrate the specific regulatory evidences of innate immune responses by three novel serpins.

In Drosophila, pathogens are recognized by circulating pattern recognition proteins that trigger the activation of the Toll signaling cascade, leading to the processing of pro-Spaetzle to Spätzle, a ligand that activates Toll receptor (1). The Toll cascade is mainly responsive to infection by Gram-positive bacteria and fungi. Lys-type peptidoglycans (PGs) from Gram-positive bacteria and β-1,3-glucans of fungi are specifically recognized by the PG recognition protein (PGRP)-SA-Gram-negative-binding protein 1 (GNBP1) complex and by GNBP3, respectively. After recognition of a pathogen, the serine protease (SP)-mediated Toll signaling cascade is activated (2–4). This pathway induces antimicrobial peptide (AMP) gene expression through the activation of the NF-κB transcription factor (1).

Another major innate immune response in invertebrates is the melanization reaction (5). A key enzyme in melanin biosynthesis is pro-phenoloxidase (pro-PO), which is released by the hemocytes (insect blood cells) that circulate in the hemolymph (insect blood) and which is cleaved to generate active PO at the end of the SP cascade (5–7). The SPs of the Toll and melanin cascades exist as inactive zymogens in the hemolymph, analogous to components of the complement pathway found in human blood (8). Because these SP cascades in vertebrates and invertebrates have significant signal amplification functions, these systems are advantageous for detecting the presence of minute levels of pathogens. However, tight regulation is necessary to prevent excessive activation of these pathways under normal conditions and to localize their actions both temporally and spatially.

Recently, we analyzed the Toll signaling SP cascade using a large beetle, Tenebrio molitor (9–11). The size of this insect enabled us to collect enough hemolymph to purify SPs by biochemical methods. Our study demonstrated that the recognition of Lys-type PGs by the PGRP-SA-GNBP1 complex activates pro-Spaetzle via the sequential activation of three different SPs: modular serine protease (MSP), Spätzle-processing enzyme-activating enzyme (SAE), and Spätzle-processing enzyme (SPE) (10). SPE has been identified as a terminal SP that cleaves pro-Spaetzle. Additionally, we provided biochemical evidence of the mechanism by which the GNBP3-mediated β-1,3-glucan recognition signal is transferred to pro-Spaetzle, leading to the production of AMPs (11). Furthermore, the terminally

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2 The abbreviations used are: PG, peptidoglycan; PGRP, PG recognition protein; AMP, antimicrobial peptide; Boc, β-1,3-butyloxycarbonyl; GNBP, Gram-negative-binding protein; MCA, 4-methylcumarin-7-amide; MSP, modular serine protease; PO, phenoloxidase; RCL, reactive center loop; SAE, Spätzle-processing enzyme-activating enzyme; SPE, Spätzle-processing enzyme; SP, serine protease; Tricine, N-[2-hydroxy-1,1-bis(hydroxy-

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activated SPE was also shown to convert the 79-kDa Tenebrio pro-PO into PO to generate a melanin complex with SP homolog 1 that is necessary for the production of melanin pigment on the bacterial cell surface, leading to a strong bactericidal effect (7).

The serpins belong to a superfamily of SP inhibitors that act as suicide substrates by binding covalently to their target proteases (12). Serpins are known to regulate various physiological processes and defense reactions in mammals (13). To date, four Drosophila serpins related to innate immunity, SPN43Ac, SPN27A, SPN77Ba, and SPN28D, have been analyzed in detail by genetic approaches. SPN43Ac mutant flies accumulated cleaved Spätzle, resulting in constitutive activation of the Toll pathway and expression of AMPs (14). SPN27A and SPN28D are known to regulate the Toll pathway during early development (15–17) and are also involved in the melanin biosynthesis reaction (18, 19). Another serpin, SPN77Ba, was identified as a negative regulator of melanization in the respiratory system (the trachea) of Drosophila (20). From Manduca sexta, six different serpins were identified and characterized as negative regulators of the pro-PO cascade (6). All of these serpins were suggested to regulate the Toll or melanization cascades. However, the molecular identities of the serpin target SPs and the biochemical regulatory mechanisms of these serpins were not clearly demonstrated, resulting in a lack of molecular understanding of the roles of serpins in the Toll and melanin synthesis cascades.

Because we identified three SPs that are directly involved in the activation of the Tenebrio Toll cascade, we assumed that Tenebrio larvae would be a useful system to identify and characterize novel target serpins that directly regulate the Toll signaling cascade. Here, we have identified three novel serpin-protease pairs that negatively regulate the Toll and melanin synthesis cascades.

**EXPERIMENTAL PROCEDURES**

**Animals and Collection of Hemolymph—**T. molitor larvae (mealworms) were maintained in terraria containing wheat bran. Hemolymph was collected as described previously (21). Briefly, to harvest the hemolymph, a larva was pricked using a 25-gauge needle, and a 10-μl drop of hemolymph was collected in 500 μl of a modified anticoagulation buffer (136 mM trisodium citrate, 26 mM citric acid, 20 mM EDTA, and 15 mM sodium chloride, pH 5.0). The collected crude hemolymph was centrifuged at 20,000 g for 15 min at 4 °C. The supernatant was then stored at −80 °C until use.

**Measurements of Melanin Synthesis**—Melanin synthesis was measured according to a method published previously (7). Briefly, 30 μl of crude hemolymph (350 μg of proteins) was preincubated for 10 μl of β-1,3-glucan (1 μg) or soluble Lys-PG (100 ng) at 30 °C for 10 min. After incubation, 460 μl of the substrate solution (2 μl Tris–HCl, pH 8.0, containing 1 mM dopamine and 10 mM CaCl2) was added to the reaction mixture and then incubated at 30 °C for 1 h. The increase in absorbance at 400 nm, which records melanin formation, was measured.

**Purification of Toll Cascade-related Proteins**—The native and recombinant forms of the PGRP-SA, GNB1, GNB3, MSP, SAE, SPE, and pro-Spätzle proteins were obtained as described previously (10, 11).

**Purification and cDNA Cloning of Serpins**—The detailed purification methods of Tenebrio SPN40, SPN55, and SPN48 are described under supplemental “Methods” S1–S6. The detailed procedures of cDNA cloning of four serpins are described under supplemental “Methods” S7. The baculoviruses to express these recombinant serpins were generated according to the manufacturer’s instructions (Invitrogen). Detailed procedures are described under supplemental “Methods” S8.

**Measurement of the Amidase Activity Generated after Activation of Three Toll Cascade-activating SPs**—To determine amidase activity in the samples, commercially available trypsin substrate (t-butoxyoxycarbonyl-benzyl-1-phenylalanyl-1- seryl-l-arginine-4-methylcoumaryl-7-amide (Boc-Phe-Ser-Arg-MCA)) was used as described previously (9). Briefly, synthetic substrate was dissolved in dimethylformamide according to the instructions from the manufacturer. One hundred μl of reaction mixture for measuring protease activity of the activated SPE was incubated with 490 μl of substrate solution, which contains 40 μM substrate in 20 mM Tris–HCl buffer, pH 8.0. After incubation of the mixture at 30 °C for 1 h, 500 μl of 17% (v/v) acetic acid was added to terminate the enzyme reaction. The specific amidase activity of the eluate solution can be detected by a fluorescence spectrophotometer at λex = 380 nm and λem = 460 nm. As a control, 100 μl of buffer A was added to check the amidase activity as above. One unit of the amidase activity was defined as the amount that liberated 1 nmol of 7-amino-4-methylcoumarin/min.

**In Vitro Reconstitution Experiments for Examining the Effects of Serpins on Amidase Activity and on Pro-Spätzle Processing**—To examine whether purified serpin(s) can inhibit the amidase activity of activated SPE, in vitro reconstitution experiments were performed with the seven components: Lys-type PG (100 ng), 100 ng of each protein (PGRP-SA, GNB1, pro-MSP, pro-SAE, pro-SPE), and Ca2+ (10 mM). These components were incubated for 5 min in the absence or presence of serpin(s) as described previously (10). The amidase activity of activated SPE was measured using the Boc-Val-Pro-Arg-MCA fluorescence substrate. To determine whether or not Spätzle processing was inhibited by serpin(s), in vitro reconstitution experiments were performed with seven components and pro-Spätzle (300 ng). Processed Spätzle (12 kDa) was examined by Western blotting analysis with anti-Spätzle antibody as described previously (10).

**Immunoblot Analysis of Serpin Induction after Injection of β-1,3-Glucan, Pro-Spätzle, and Processed Spätzle**—To determine which serpin(s) is induced in the hemolymph, four components, insect saline (4 μl), β-1,3-glucan (50 ng), recombinant pro-Spätzle (60 ng), and processed Spätzle (60 ng), were injected into Tenebrio larvae, and then hemolymph collected after 24 h was analyzed to determine the amounts of serpin(s) by immunoblot analysis using each anti-serpin antibody.

**Determination of the Cleavage Sites of SPN40, SPN55, and SPN48 by Activated MSP, SAE, and SPE**—We followed the previously published method with little modification (22). We prepared SPN40-MSP, SPN55-SAE, and SPN48-SPE complexes by co-incubation of the serpins and the target SPs. After incuba-
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tion, the reaction mixture was separated using SDS-PAGE under reducing conditions. The N-terminal sequences of released C-terminal fragments of serpins during formation of SP-serpin complexes were analyzed. Briefly, a complex was formed by incubating 100 pmol of recombinant SPN40, SPN55, or SPN48 and 50 pmol of activated MSP, SPE, or SAE for 4 h at room temperature in 150 μl of 20 mM Tris-HCl, pH 7.8. The complex mixture (200 μl) was mixed with SDS-PAGE loading buffer and then loaded onto a Tricine SDS-polyacrylamide gel. After running, the gels were blotted onto polyvinylidene difluoride membranes and then stained with a solution containing 0.1% Coomassie Brilliant Blue R-250 in 40% methanol. The membrane was then destained with 50% methanol, the bands corresponding to the released C-terminal fragments were cut from the membrane, and the N-terminal sequences of the fragments were determined using an automatic amino acid sequencer.

RESULTS

Three Purified Serpins Formed Specific Complexes with Three Toll Cascade-activating SPs—A hallmark of the specific inhibition of SPs by serpins is the formation of a covalently bonded SP-serpin complex that can be seen as a high molecular mass band using SDS-PAGE (23), the identity of which is easily confirmed by SP antibodies (24). Because we have already obtained active forms of three Toll-cascade activating SPs (lanes 1, 4, and 7 in Fig. 1A) and raised polyclonal antibodies to these proteins in our recent study (10), we decided to monitor the generation of SP-serpin complexes to purify serpins. Using this method, we succeeded in purifying three novel serpins with molecular masses of 40, 55, and 48 kDa (named SPN40, SPN55, and SPN48, respectively) as determined by SDS-PAGE (lanes 2, 5, and 8 in Fig. 1A). These proteins formed SP-serpin complexes with MSP, SAE, and SPE, respectively (indicated by arrows in lanes 3, 6, and 9 in Fig. 1A). To examine the specificity of each serpin for its target SP in vitro, we performed immunoblot analyses after incubation of each purified serpin with the active forms of the three Toll-cascade-activating SPs (Fig. 1B). As expected, SPN40 only formed a complex with activated MSP (lane 2); it did not form a complex with activated SAE or SPE (lanes 3 and 4). Also, SPN55 and SPN48 only formed complexes with the active forms of SAE and SPE, respectively (indicated by arrows in lanes 3, 6, and 9 in Fig. 1A). To examine the specificity of each serpin for its target SP in vitro, we performed immunoblot analyses after incubation of each purified serpin with the active forms of the three Toll-cascade-activating SPs (Fig. 1B). As expected, SPN40 only formed a complex with activated MSP (lane 2); it did not form a complex with activated SAE or SPE (lanes 3 and 4). Also, SPN55 and SPN48 only formed complexes with the active forms of SAE and SPE, respectively (indicated by arrows in lanes 3, 6, and 9 in Fig. 1A). To examine the specificity of each serpin for its target SP in vitro, we performed immunoblot analyses after incubation of each purified serpin with the active forms of the three Toll-cascade-activating SPs (Fig. 1B). As expected, SPN40 only formed a complex with activated MSP (lane 2); it did not form a complex with activated SAE or SPE (lanes 3 and 4). Also, SPN55 and SPN48 only formed complexes with the active forms of SAE and SPE, respectively (indicated by arrows in lanes 3, 6, and 9 in Fig. 1A).

FIGURE 1. Three Tenebrio serpins formed specific complexes with their target SPs. A, the specific SP-serpin complexes were observed using SDS-PAGE. The purified activated Toll cascade-activating SPs (500 ng, lanes 1, 4, and 7) and purified serpins (1 μg, lanes 2, 5, and 8) were incubated for 4 h at 30 °C and then analyzed by SDS-PAGE under reducing conditions. The gels were stained with a solution containing 0.1% Coomassie Brilliant Blue (CBB) R-250. Arrows in lanes 3, 6, and 9 indicate the SP-serpin complex. Star and arrowheads indicate the N-terminal domain of MSP and the C-terminal catalytic SP domains, respectively. Triangles indicate cleaved SPN40 and SPN55. B, each purified serpin (20 pmol, including SPN40 (lanes 1–4), SPN55 (lanes 5–8), SPN48 (lanes 9–12), and SPN1 as a control (lanes 13–16), was incubated with a Toll cascade-activating SP (5 pmol), MSP, SAE, or SPE, under the same conditions as in A. The reaction mixtures were analyzed by immunoblotting to visualize the SP-serpin complexes.

Determination of Actual Cleavage Sites of SPN40, SPN55, and SPN48 by Activated SPs—In our recent studies, we demonstrated that MSP has chymotrypsin-like catalytic enzyme activity, whereas SAE and SPE have trypsin-like enzyme activity (10). The putative P1 residues of SPN40, SPN55, and SPN48 were predicted to be Pro, Met, and Pro, respectively, based on the alignment of the deduced amino acid sequences (Fig. 2). Even though the putative P1 Pro or the Leu residue at either P2 or P1’ of SPN40 is a possible target site for chymotrypsin-like MSP, the P1 Met residue of SPN55 and the P1 Pro residue of SPN48 are unusual target residues for the trypsin-like SAE and SPE proteases. To examine whether these putative P1 residues are actually cleaved by the target chymotrypsin-like MSP, trypsin-like SAE, or SPE during formation of the SP-serpin complexes, we purified the C-terminal fragments released from SPN40, SPN55, and SPN48 during complex formation, and the
Production of the SPN40 and SPN55 Serpins in Vivo—When six Toll cascade-regulating factors, such as PGRP-SA, GNBP1, pro-MSP, pro-SAE, pro-SPE, and Ca\(^{2+}\), were co-incubated with Lys-type PG for 5 min, activated SPE showed amidase activity (column 2). When purified serpin was added to column 2, the amidase activity gradually decreased. This was the case for 500 ng of each SPN40 (column 3), SPN55 (column 4), and SPN48 (column 5) and for the mixture of all three serpins (column 6). A, when six Toll cascade-regulating components (each 100 ng) and pro-SPE were incubated with Lys-type PG for 5 min as in A, processed Spätzle (12 kDa) was observed by Western blotting with an affinity-purified anti-Spätzle antibody (lane 2). When serpin was added to the reaction mixture, pro-Spätzle processing was gradually inhibited by SPN40 (lane 3), SPN55 (lane 4), SPN48 (lane 5) and the mixture of the three serpins (lane 6). The 30-kDa pro-Spätzle and the 12-kDa processed Spätzle are indicated with arrows. T-bars mean ± S.D. (p < 0.05) of three independent experiments.

**Figure 3. Three serpins inhibited Lys-type PG-mediated amidase activity and blocked the conversion of pro-Spätzle to processed Spätzle.**

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**Figure 2. Sequence alignment of the putative and experimentally determined cleavage sites (P1-P1') in the serpins RCL domains.** Blue boxes indicate the hinge region (P15-P9) of the RCL domains. Blue highlighted residues are the putative P1 sites cleaved by target SPs. Red arrows indicate the experimentally determined cleavage sites of Dm-SPN4 (30), Ms-SPN6 (31), human antithrombin (32), and C1 inhibitor (22) by their target SPs. Red arrows indicate the experimentally determined cleavage sites of Dm-SPN4 (30), Ms-SPN6 (31), human antithrombin (32), and C1 inhibitor (22) by their target SPs. Spätzle was completely converted into cleaved Spätzle after 5 min in the absence of serpins (lane 2). As expected, this processing was also gradually inhibited by the addition of SPN40, SPN55, and SPN48 individually (lanes 3–5). Finally, addition of all three serpins together completely inhibited the processing of pro-Spätzle to its mature form (lane 6). These results demonstrate that the three serpins make irreversible SP-serpin complexes with their target SPs and then inhibit the activation of the three-step proteolytic cascade, leading to the blockage of the Spätzle processing.

Injection of Processed Spätzle into Tenebrio Larvae Increased Production of the SPN40 and SPN55 Serpins in Vivo—When invading pathogens are recognized by the soluble pattern recognition proteins in the hemolymph, SPzymogens upstream of the Toll cascade are sequentially converted to the active forms
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of the SPs to amplify the pathogen recognition signals (10, 11, 26). We assumed that endogenous upstream serpin(s) present in small amounts in unstimulated conditions could also be induced as a negative feedback inhibitor in vivo coincident with Toll cascade activation. To prove this hypothesis, we injected β-1,3-glucan as an initial activator of the Toll cascade and pro-Spätzle or the processed Spätzle as a ligand of the Toll receptor into Tenebrio larvae and then examined an increase in protein level of serpin(s) by immunoblot analysis. As expected, the levels of SPN40 and SPN55, but not those of SPN48 or SPN1, were dramatically increased 24 h after injection of β-1,3-glucan and processed Spätzle (lanes 3 and 5 in Fig. 4). Pro-Spätzle also induced reduced amount of SPN40 (lane 4), which may be attributed to the autoactivation of pro-Spätzle in the hemolymph. These results strongly support the hypothesis that endogenous SPN40 and SPN55 are induced to eliminate the activated MSP and SAE for the precise regulation of the Toll cascade in the hemolymph.

**Serpins Blocked β-1,3-Glucan-induced Melanin Synthesis in Vivo**—Next, we tried to determine the roles of these serpins in melanin synthesis, which is triggered by activated SPE generated after sequential activation of the common three-step SP cascade (7). When we injected a mixture of the three serpins and β-1,3-glucan into Tenebrio larvae, melanin synthesis was greatly decreased compared with synthesis in control groups treated only with β-1,3-glucan (Fig. 5A). In addition, the survival rate was improved by co-injection of the three serpins and β-1,3-glucan (Fig. 5B). These results demonstrate that these *Tenebrio* serpins efficiently inhibit the activation of both the SP-mediated Toll and the melanin synthesis cascades in vivo. To further confirm these results in vitro, we examined whether the conversion of pro-PO to PO, which is catalyzed by activated SPE, is inhibited by SPN48 or not (Fig. 5C). When the mixture of pro-PO, SP homolog 1 zymogen, and activated SPE was incubated together with dopamine, melanin complexes were generated (lane 4), as reported previously (7). Addition of increased amounts of SPN48 to the mixture inhibited the generation of the melanin complex (lanes 5–7). These results also support the hypothesis that SPN48 inhibits the enzymatic activity of SPE, leading to the inhibition of the processing of pro-PO and the inhibition of melanin synthesis in vitro.

**DISCUSSION**

A carefully regulated balance between activation and inhibition of SP-mediated innate immune responses must be maintained to avoid detrimental damage to the host (4). In this study, we identified three serpins that target three Toll
cascade-activating SPs and act as negative regulators of the Toll signaling and melanin synthesis cascades. This is the first determination and functional study of specific SP-serpin pairs that are directly involved in the regulation of the pattern recognition protein-dependent Toll signaling cascade.

Contrary to expectations, the activity of each protease in the proteolytic cascade is regulated by a specific serpin inhibitor. It had been thought that such cascades were regulated by the activity of a single "bottleneck" protease. There are increasing indications, in both mammals and the Drosophila model system, that control of proteolytic signaling cascades may be far more precisely regulated than previously thought. Our article presents the first indication that each individual protease in a cascade may be regulated by a specific serpin. Furthermore, we have shown that SPN40 and SPN55 function as inducible negative feedback regulators of the Toll cascade. Also, the three serpins blocked activation of pro-PO, leading to inhibition of melanin synthesis.

Because activated SAE and SPE are known to cleave after Arg residues in the downstream SPEzymogen (Arg199-Ile200) and pro-Spätzle (Arg124-Phe125), respectively, during the activation of the Tenebrio Toll cascade (10), we expected that SPN55 and SPN48 might have an Arg residue in the P1 position. However, SPN55 is cleaved between the putative P2 Tyr and P1 Met residues by the trypsin-like SAE, and the cleavage site of SPN48, which is targeted by the trypsin-like SPE, was determined to be between the putative P1’ Glu and P2’ Met residues. These unusual P1 specificities of the Arg-targeting SPs have also been observed in several mammalian serpins (12, 27, 28). For example, kallikrein, a serpin that inhibits tissue kallikrein, has a P1 Phe residue, despite the fact that kallikrein targets Arg-specific substrates (28). Also, protein Z-dependent protease inhibitor, which has a P1 Tyr residue, is a specific inhibitor of membrane-bound factor Xa, an Arg-specific SP (12).

These unexpected findings provide us with another example of the enhancement of specificity and of the tight regulation of the Toll cascade by insect endogenous serpins, which are co-localized with many different digestive SPs in the insect circulatory system. Combinations of the three different serpins described in this study will enhance the high specificity of the proteolytic Toll signaling cascade and tightly regulate the activation of this cascade. Our study also provides novel insights into the precisely regulated pathogen recognition mechanisms of innate immune responses.

In this study, even though we injected purified three serpins into Tenebrio larvae several times to examine the blockage of the production of AMPs as effector molecules of Toll cascade activation, it was impossible to observe the inhibition of AMP production (data not shown). The reason was attributed to the fact that Drosophila has alternative defense signaling pathways, such as danger signaling or Imd pathway (4, 29), leading to production of AMPs despite Toll cascade blockade. However, under the same conditions, we observed that melanin synthesis cascade was inhibited by injection of three serpins (Fig. 5), suggesting that AMP synthesis might be controlled by branched alternative pathway in vivo, but melanin synthesis might be controlled by the one SP pathway in vivo.

In summary, we present here the biochemical analysis data for three novel serpins that regulate Toll signaling and melanin synthesis cascades in vivo and in vitro. Our data demonstrate for the first time the regulatory mechanisms of pattern recognition protein-mediated Toll signaling cascade by three endogenous novel serpins, which are specifically making SP-serpin complexes with three Toll cascade-activating SPs. This study further highlights the elaborative regulatory mechanism and the complexity of invertebrate major defense reactions that can be differently regulated toward different SP during activation of Toll and melanin synthesis cascades.

REFERENCES
Toll Cascade-regulating Serpins