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Molecular Control of Phenoloxidase-induced Melanin Synthesis in an Insect*[S]◆

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The melanization reaction induced by activated phenoloxidase in arthropods must be tightly controlled because of excessive formation of quinones and excessive systemic melanization damage to the hosts. However, the molecular mechanism by which phenoloxidase-induced melanin synthesis is regulated *in vivo* is largely unknown. It is known that the Spätzle-processing enzyme is a key enzyme in the production of cleaved Spätzle from pro-Spätzle in the *Drosophila* Toll pathway. Here, we provide biochemical evidence that the *Tenebrio molitor* Spätzle-processing enzyme converts both the 79-kDa *Tenebrio* prophenoloxidase and *Tenebrio* clip-domain SPH1 zymogen to an active melanization complex. This complex, consisting of the 76-kDa *Tenebrio* phenoloxidase and an active form of *Tenebrio* clip-domain SPH1, efficiently produces melanin on the surface of bacteria, and this activity has a strong bactericidal effect. Interestingly, we found the phenoloxidase-induced melanization reaction to be tightly regulated by *Tenebrio* prophenoloxidase, which functions as a competitive inhibitor of melanization complex formation. These results demonstrate that the *Tenebrio* Toll pathway and the melanization reaction share a common serine protease for the regulation of these two major innate immune responses.

The *Drosophila* Toll signaling pathway is responsible for defending against Gram-positive bacteria and fungi by inducing the expression of antimicrobial peptides via NF-κB-like transcription factors (1, 2). The recognition of lysine-type peptidoglycan (PG)[2] by the *Drosophila* PG recognition protein-SA and GNBP1 (Gram-negative binding protein 1) complex has been suggested to cause activation of the serine protease cascade, leading to the processing of Spätzle and subsequent activation of the Toll signaling pathway (2, 3). The elegant genetic studies in *Drosophila* have been and remain very powerful for characterizing and arranging the components of the *Drosophila* Toll pathway (4). Recently, we found that three serine proteases are involved in the activation of the Toll pathway in a large beetle, *Tenebrio molitor*, and we indicated the sequence in which they are activated *in vitro* (5). This three-step proteolytic cascade linking the PG recognition complex and subsequent Spätzle processing is essential for the peptidoglycan-dependent Toll signaling pathway (5).

The prophenoloxidase (proPO) activation cascade is known to be one of the major innate immune responses in arthropods (6–8), even though the role of the proPO activation cascade remains controversial in *Drosophila* and mosquito innate immunity (9, 10). Upon injury or infection, proPO in the blood plasma is activated to phenoloxidase (PO) by clip-domain serine proteases, which are called proPO-activating factors (PPAFs) or enzymes (PPAEs), or alternatively proPO-activating proteins (11–14). We recently determined the crystal structures of two PPAFs and the functional roles of the clip domains during the proPO activation cascade (15, 16). PO, the active form of proPO, catalyzes the production of quinones, which can nonspecifically cross-link neighboring molecules to form melanin at the injury site or all over the surface of invading microorganisms (17, 18). Quinones may also be involved in the production of cytotoxic molecules such as superoxides and hydroxyl radicals, which could help to kill the invading microorganisms (6, 19). PO-induced melanin synthesis is thought to be essential for defense and development, but it must be tightly controlled because systemic hyperactivation of the proPO system, excessive production of quinones, and excessive melanin synthesis are harmful to the host. This implies that proPO activation and melanin synthesis are tightly regulated by melanization regulatory molecules (6, 7). However, the molecular regulatory mechanism of melanin synthesis is unclear.

Recently, we reported that a soluble fragment of Lys-type PG, a long glycan chain with short stem peptides, is a potent activa-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Experimental Procedures," Fig. 1, and additional references.

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[2] The abbreviations used are: PG, peptidoglycan; proPO, prophenoloxidase; PO, phenoloxidase; PPAF, proPO-activating factor; PPAE, proPO-activating enzyme; Tm, *T. molitor*; PGRP-SA, PG recognition protein-SA; MSP, modular serine protease; SPH, serine protease homologue; SPE, Spätzle-processing enzyme.

tor of the *Drosophila* Toll pathway and the *Tenebrio* proPO activation cascade (20). The fact that the same elicitor activates both the proPO system and the Toll pathway and that the clustering of *T. molitor* (Tm) PG recognition protein-SA (PGRP-SA) molecules on the PG is followed by activation of the proPO cascade suggests that there are obvious possibilities for molecular cross-talk between these two innate immune responses. Consistent with this possibility, we showed that partial lysozyme digestion of highly cross-linked Lys-type PG dramatically increases the binding of Tm-PGRP-SA, presumably by inducing clustering of Tm-PGRP-SA, which then recruits Tm-GNBP1 and Tm-modular serine protease (MSP) (20). In that study, we suggested that formation of the Lys-type PG·Tm-PGRP-SA·Tm-GNBP1 complex leads to the activation of downstream PPAEs or PPAFs. However, we did not investigate the detailed molecular cross-talk between the proPO and Toll cascades.

We and other groups have reported the biochemical properties of clip-domain serine protease and clip-domain serine protease homologues (SPHs) that function as PPAEs or PPAFs (21–25). All these serine proteases are known to consist of a trypsin-like domain at the C terminus and one or two clip domains at the N terminus, but all SPHs lack proteolytic activity due to substitution of the catalytic Ser residue with a Gly residue (16). We reported the detailed characterization of three PPAFs purified from the larvae of a large beetle, *Holotrichia diomphalia* (26). Two of these PPAFs have been crystallized, and these structural studies have provided details about the activation mechanism (15, 16). For example, they showed that proPO cleavage alone is insufficient to produce active PO. In fact, an activated SPH (reported to be PPAF-II) is needed to lead to enzymatically active PO. Furthermore, the structural studies showed that when an active PPAF cleaves an SPH, the activated SPH oligomerizes, and the clip domain of SPH acts as a module for binding PO (16). This probably serves to ensure that the active PO is under tight control and does not spread through the hemolymph in an uncontrolled manner but rather remains in the vicinity of the original trigger of the activation cascade. These findings provided some of the clues about how the proPO cascade and melanin synthesis are tightly regulated, but they did not provide detailed insights.

To provide compelling biochemical evidence for the regulatory control of melanin synthesis and to search for possibilities of molecular cross-talk between the Toll pathway and proPO activation, we purified Tm-proPO, Tm-Spätzle-processing enzyme (SPE), and two different SPH zymogens (Tm-SPH1 and Tm-SPH2) to homogeneity. By performing *in vitro* reconstitution experiments with these purified proteins, we provide clear biochemical evidence that the active form of Tm-SPE and a specific Tm-SPH1 tightly regulate the activation of proPO and melanin synthesis in the Tm-proPO system.

EXPERIMENTAL PROCEDURES

Insect and Antibodies—T. molitor larvae (mealworm) were maintained on a laboratory bench in terraria containing wheat bran. Hemolymph was collected as described previously (27). Briefly, to harvest the hemolymph, a larva was pricked using a 25-gauge needle, and then a 10-μl drop of hemolymph was collected in 50 μl of 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 200,000 $\times$ *g* for 15 min at 4 °C. The supernatant was then stored at −80 °C until used. Rabbit antisera against Tm-proPO, Tm-SPE, and Tm-SPH1 raised previously (5, 21, 28) were used after affinity purification as described previously.

Determination of Melanin Synthesis—Estimation of PO-induced melanin synthesis was carried out according to our previously published method (29). In brief, a mixture of the active form of Tm-SPE (150 ng), Tm-proPO (3 μg), and Tm-SPH1 zymogen (1 μg) in 50 μl of 20 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$ was preincubated at 30 °C for 5 min, after which 150 μl of the substrate solution (20 mM Tris-HCl, pH 8.0, containing 1 mM dopamine and 10 mM $CaCl_2$) were added, and the mixture was incubated at 30 °C for 30 min. The increase in absorbance at 400 nm, which occurred in parallel with the synthesis of melanin, was measured using a Shimadzu spectrophotometer.

Purification of Tm-proPO, Active Tm-SPE, and Tm-SPH2 Zymogen—Detailed procedures used to purify Tm-proPO, active and zymogenic forms of Tm-SPE, and Tm-SPH2 from the hemolymph (insect blood) of *T. molitor* larvae are described under the supplemental "Experimental Procedures."

In Vitro Reconstitution Experiments and Peptide Sequencing—To determine the cleavage sites of Tm-proPO and Tm-SPH1 induced by active Tm-SPE, *in vitro* reconstitution experiments were performed as described previously (5). To map proteolytic cleavage sites, the reaction mixtures following enzyme treatment were analyzed by SDS-PAGE under reducing conditions, blotted onto a polyvinylidene difluoride membrane, and stained with a solution containing 0.1% Coomassie Brilliant Blue R-250 and 50% methanol. The membrane was destained with 50% methanol containing 10% (v/v) acetic acid until the protein bands became visible. The zymogen cleavage products were identified by their N-terminal sequences using a gas-phase protein sequencer (Applied Biosystems).

Immunofluorescence Microscopy—Melanin-coated bacterial cells were fixed in ice-cold 100% methanol. After washing twice with ice-cold phosphate-buffered saline, bacteria were incubated in phosphate-buffered saline containing 0.3% Tween 20 (blocking buffer) containing 3% skim milk for 1 h. After washing, cells were sequentially incubated with mouse anti-His monoclonal antibody (H-3, Santa Cruz Biotechnology) and rabbit anti-Tm-proPO polyclonal antibody (1:100 in blocking buffer) for 2 h. Fluorescein isothiocyanate-conjugated goat anti-mouse antibody or rhodamine-conjugated goat anti-rabbit antibody (1:200 in blocking buffer; Santa Cruz Biotechnology) was then added and incubated for 1 h. After washing twice with blocking buffer, stained cells were observed under a Zeiss fluorescence microscope.

Expression and Purification of Recombinant Tm-SPH1—A DNA fragment encoding Tm-SPH1 (21) was amplified by PCR using primers 5′-CCCGGATTCGCAAAAAGATGTCGATGATGCT-3′ and 5′-CCCTCTAGATCATATCAGGTAAGAGGATGTACCA-3′ with BamHI and XbaI at the 5′ and 3′ termini, respectively. These sites were later used to add a C-terminal tobacco etch virus protease cleavage site and a hexahis-

tidine tag. The PCR products were subcloned into the pFastBac-SEa vector. The resulting plasmid was transformed into DH10Bac cells, and the transformation mixture was spread on LB agar culture medium containing isopropyl β-D-thiogalactopyranoside, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), gentamicin, kanamycin, and tetracycline. The white colonies were selected and cultured for amplification, and the bacmid DNA was extracted. After bacmid verification, Sf9 cells were transfected with the bacmid DNAs, and the resulting viruses were harvested and amplified as recommended by Invitrogen (Bac-to-Bac baculovirus expression system manual). For protein expression, Sf9 cells in 1 liter of suspension culture were infected with recombinant baculovirus. For purification of the expressed protein, the medium was first concentrated to ~100 ml by ultrafiltration through a membrane filter and then dialyzed at 4 °C against 10 liters of 20 mM Tris-HCl and 150 mM NaCl, pH 8.0. After centrifugation at 20,000 × *g* for 15 min at 4 °C to remove precipitates, the supernatant was mixed with 3 ml of precharged nickel-nitrilotriacetic acid-agarose (Qiagen Inc.) and gently rotated at 4 °C for 1 h. Unbound proteins were allowed to pass through the resin, which was then washed with 200 ml of the same buffer containing 20 mM imidazole. Bound proteins were eluted with 200 mM imidazole in the same buffer and analyzed by SDS-PAGE under reducing conditions. For His tag removal, the fractions containing expressed proteins were incubated with tobacco etch virus protease (Invitrogen). His-tagged removed recombinant Tm-SPH1 was purified using a size exclusion high pressure liquid chromatography column (TSK G3000SW) equilibrated with 50 mM Tris-HCl containing 150 mM NaCl, pH 8.0. The fractions containing expressed proteins were pooled and concentrated on Centricon YM-10 to a final concentration of 0.1 mg/ml. The N-terminal amino acid sequencing of the recombinant Tm-SPH1 protein was carried out to verify the identities of the purified proteins.

Inhibition Experiments Using Tm-proPO—The reaction mixture containing Tm-proPO (3 μg), Tm-SPH1 zymogen (1 μg), and the active form of Tm-SPE (150 ng) in 50 μl of buffer (20 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$) was incubated at 30 °C for 60 min, and the reaction mixtures were cooled on ice for 5 min and divided into three equal portions. One portion was mixed with 200 μl of substrate solution (20 mM Tris-HCl, pH 8.0, containing 1 mM dopamine and 10 mM $CaCl_2$) at 30 °C for 15 min, and the melanin synthesis activity was estimated. Benzamidine was added to the second portion to a final concentration of 5 mM; the mixture was incubated at 37 °C for 1 h; and the melanin synthesis activity was examined. Benzamidine to a final concentration of 5 mM and 30 μg of Tm-proPO were simultaneously added to the third portion; the mixture was incubated at 37 °C for 1 h; and the melanin synthesis activity was estimated as described above.

Assay of Antibacterial Activity—Antibacterial activity was assayed essentially as described previously (30). Briefly, the bactericidal activity of melanin-concentrated bacteria was assayed using *Staphylococcus aureus* (strain Cowan 1) and *Escherichia coli* (strain K12). Bacteria grown in antibiotic medium were collected during the exponential phase of growth and suspended in 10 mM sodium phosphate buffer containing 130 mM NaCl, pH 6.0. The bacterial cells (10^6) were incubated with a mixture of active Tm-SPE (150 ng), Tm-proPO (3 μg), and Tm-SPH1 (1 μg) in the presence of 1 mM L-dopamine in 200 μl of assay buffer (20 mM Tris-HCl, pH 8.0, and 10 mM $CaCl_2$) for 40 min at 30 °C. Melanin-coated bacteria were diluted 500-fold with assay buffer, and aliquots of 50 μl were spread on Bacto-agar (Difco). The plates were incubated for 18 h at 37 °C, and colony numbers on test and control plates were compared.

RESULTS

Active Tm-SPE Induces Melanin Synthesis in Vivo—Recently, we reported that the active form of Tm-SPE cleaves the 24-kDa Spätzle proprotein between Arg^{124} and Phe^{125} residues (5). Cleaved 14-kDa Spätzle, a ligand of the Toll receptor, induces strong antimicrobial activity when injected into *Tenebrio* larvae (5). In addition, we observed previously that an unidentified serine protease specifically cleaved 79-kDa Tm-proPO between Arg^{50} and Phe^{51}, and the resulting molecule of cleaved Tm-76-kDa PO was concentrated in the residues of the melanized cell clump/adhesion region (28). To examine the possibility that active Tm-SPE can cleave Tm-proPO between Arg^{50} and Phe^{51} and thereby induce melanin synthesis *in vivo*, we injected the active form of Tm-SPE into *Tenebrio* larvae. The active form of Tm-SPE induced high levels of melanin synthesis after 4 days (Fig. 1*A*). These results suggest that the active form of Tm-SPE may convert Tm-proPO to Tm-PO, and the activated Tm-PO then induces melanin synthesis *in vivo*.

To confirm that active Tm-SPE cleaves Tm-proPO between Arg^{50} and Phe^{51} and then induces melanin synthesis *in vitro*, we incubated purified Tm-proPO (Fig. 1*B*, *lane 2*) with purified active Tm-SPE (*lane 1*) and subjected the reaction samples to SDS-PAGE under reducing conditions (*lanes 3* and *4*). Surprisingly, active Tm-SPE generated three new bands (bands a–c). N-terminal sequencing of these three newly generated bands (Fig. 1*C*) revealed sequences identical to those deduced for Tm-proPO zymogen (28). This result suggests that 79-kDa Tm-proPO was cleaved by active Tm-SPE at two different sites: one at Arg^{51} and another at Arg^{281} (Fig. 1*D*). To examine whether the cleaved products of Tm-proPO induce melanin synthesis in the presence of active Tm-SPE, the mixture was incubated with L-dopamine. This mixture did not induce any melanin production (Fig. 1*E*, *bar 2*); given that we observed melanin synthesis *in vivo*, this result indicates that some other molecule(s) may be required to induce melanin synthesis *in vitro*.

Because an SPH is required to form an active PO complex in *Holotrichia* (16), we selected *Tenebrio* SPHs. We reported previously that *Tenebrio* SPHs, such as 56-kDa Tm-SPH1 (referred to as Tm-mas (21)) and 52-kDa Tm-SPH2 (referred to as Tm-45 (22)), are necessary for showing PO activity in the insect *T. molitor*. However, the biological functions of these SPHs during proPO activation were not determined. When we co-incubated Tm-proPO, active Tm-SPE, and purified Tm-SPH1 zymogen, the mixture induced strong melanin synthesis (Fig. 1*E*, *bar 3*). However, if the reaction mixture contained Tm-SPH2 instead of Tm-SPH1, no melanin synthesis was induced (*bar 4*). These results suggest that a specific SPH is required for proper activation of *Tenebrio* proPO because SPH1 but not SPH2 triggers melanin synthesis.

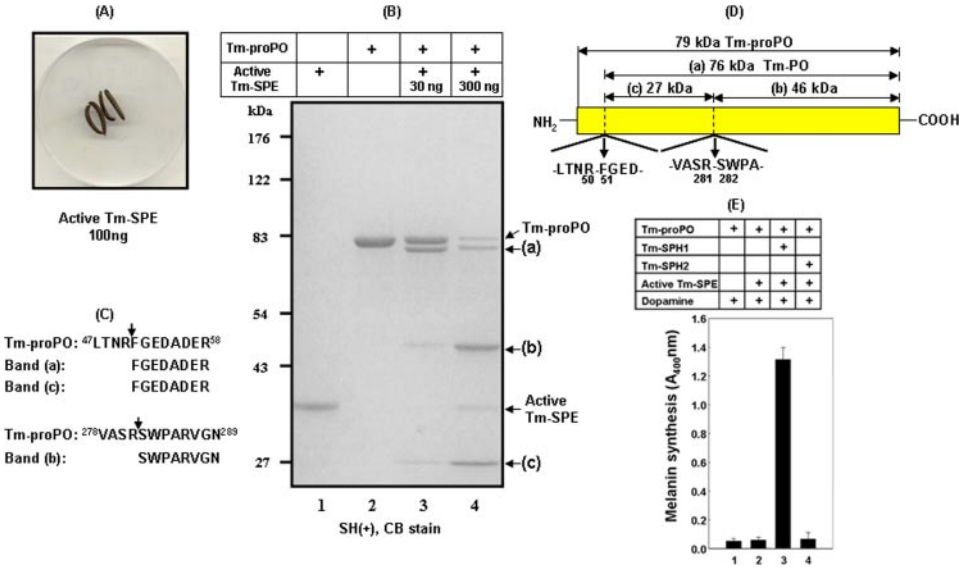

FIGURE 1. **Melanin synthesis induced by injection of active Tm-SPE and the cleavage patterns of Tm-proPO and melanin synthesis activity induced by active Tm-PO and Tm-SPHs.** *A*, melanin synthesis was estimated after injection of 100 ng of active Tm-SPE into *Tenebrio* larvae. Within 4 days, the larvae were assayed for the production of melanin pigment. *B*, shown are the SDS-PAGE analysis patterns for the cleaved products following incubation with 1 μg of Tm-proPO and 30 ng of active Tm-SPE (*lane 3*) or 300 ng of active Tm-SPE (*lane 4*). *CB*, Coomassie Brilliant Blue R-250. *C*, partial amino acid sequences were determined for the cleavage products. Bands a–c are compared with the sequence of Tm-proPO. *D*, a diagram of the sites of cleavage of Tm-proPO by active Tm-SPE is shown. *E*, *bars 1–4* show melanin synthesis activity during *in vitro* reconstitution experiments involving four different purified proteins in the presence of L-dopamine.

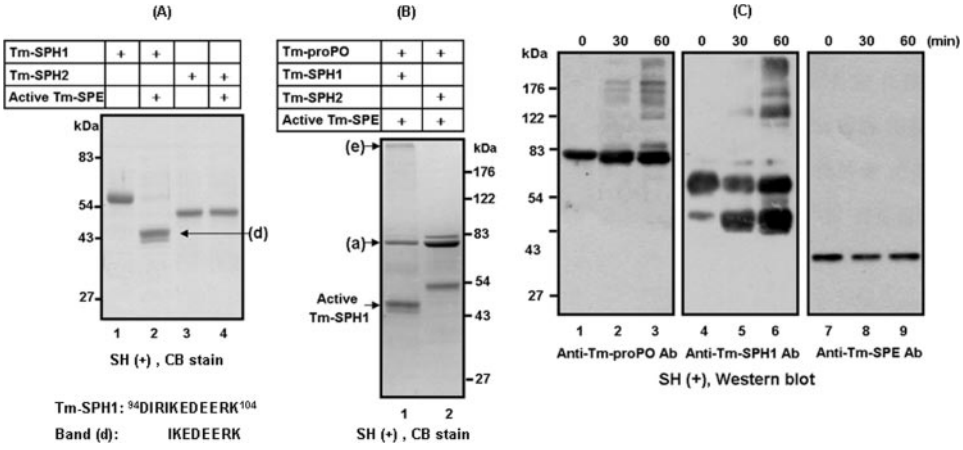

FIGURE 2. **Patterns of Tm-SPH cleavage by active Tm-SPE and analysis of the protein composition of the melanization complex.** *A*, shown are the SDS-PAGE analysis patterns for the reaction mixture after incubation of active Tm-SPE (30 ng) with Tm-SPH1 (1 μg; *lane 2*) or Tm-SPH2 (1 μg; *lane 4*). The N-terminal amino acid sequence of band d is aligned with the Tm-SPH1 sequence. *CB*, Coomassie Brilliant Blue R-250. *B*, shown are the SDS-PAGE analysis patterns for the reaction mixture of Tm-proPO (1 μg), Tm-SPH1 (1 μg), and active Tm-SPE (30 ng) (*lane 1*) and of active Tm-proPO (1 μg), Tm-SPH2 (1 μg), and active Tm-SPE (30 ng) (*lane 2*). *C*, shown is a Western blot of the reaction mixture corresponding to *lane 1* in *B* carried out using anti-Tm-proPO antibody (*Ab*; *lanes 1–3*), anti-Tm-SPH1 antibody (*lanes 4–6*), and anti-Tm-SPE antibody (*lanes 7–9*) for different incubation times.

Tm-SPE Proteolytically Cleaves Tm-SPH1—To compare the roles of Tm-SPH1 and Tm-SPH2 in melanin synthesis, we first analyzed the cleavage patterns of Tm-SPHs after incubation with active Tm-SPE *in vitro* (Fig. 2*A*). Active Tm-SPE rapidly cleaved Tm-SPH1 zymogen between Arg^{96} and Ile^{97}, generating the 43-kDa active form of Tm-SPH1 (Fig. 2*A*, *lane 2*, *band d*). Under the same conditions, however, Tm-SPH2 was not cleaved (*lane 4*). This result supports the idea that active Tm-SPE specifically cleaves Tm-SPH1 but not Tm-SPH2.

To examine whether Tm-SPH1 is involved in melanin synthesis, we compared SDS-PAGE patterns when active Tm-SPE and Tm-proPO were mixed in the presence or absence of Tm-SPH1 zymogen (Fig. 2*B*, *lanes 1* and *2*). Interestingly, only a 76-kDa band was generated in the presence of Tm-SPH1; performing SDS-PAGE under reducing conditions led to the appearance of an additional high molecular mass band (band e) at the top of the gel (Fig. 2*B*, *lane 1*). However, this band was not generated in the presence of Tm-SPH2 (*lane 2*). These results suggest that the active form of Tm-SPE completely converts 79-kDa Tm-proPO to 76-kDa Tm-PO by cleaving at the first site (Arg^{50}), but not at the second one (Arg^{281}). This cleavage subsequently led to production of the chemically cross-linked high molecular mass band (band e). To explore what kind of proteins are engaged in this high molecular mass band (band e), we performed Western blot analysis using anti-Tm-proPO, anti-Tm-SPE, and anti-Tm-SPH1 antibodies (Fig. 2*C*). Band e was recognized by antibodies against both Tm-proPO and Tm-SPH1 (Fig. 2*C*, *lanes 3* and *6*), but not by an antibody against Tm-SPE (*lane 9*). This result supports the idea that band e is a covalently cross-linked protein complex consisting of active Tm-PO and active Tm-SPH1.

Chemically Cross-linked Adducts Are Generated by the Melanization Complex—To conduct further tests of whether covalently cross-linked high molecular mass complexes consist of active Tm-PO and active Tm-SPH1, we tried to isolate this complex by size exclusion column chromatography. First, as a control, the mixture of Tm-proPO and active Tm-SPE was loaded onto a size exclusion column, and each fraction of peak 1 was analyzed by SDS-PAGE under reducing conditions (Fig. 3, *A* and *B*). As shown previously in Fig. 2*A*, the mixture of Tm-proPO cleavage products co-eluted in peak 1 (Fig. 3*B*, *lanes 2–4*). However, when we loaded the mixture of Tm-proPO, active Tm-SPE, and Tm-SPH1 zymogen onto the same column, the proteins eluted as two peaks (Fig. 3*C*). When we examined the melanin synthesis activities of the two peaks, peak 2 was quite active in synthesizing melanin, but peak 3 was not under the same conditions (Fig. 3*D*). This result suggests that peak 2 contains protein components capable of melanin synthesis.

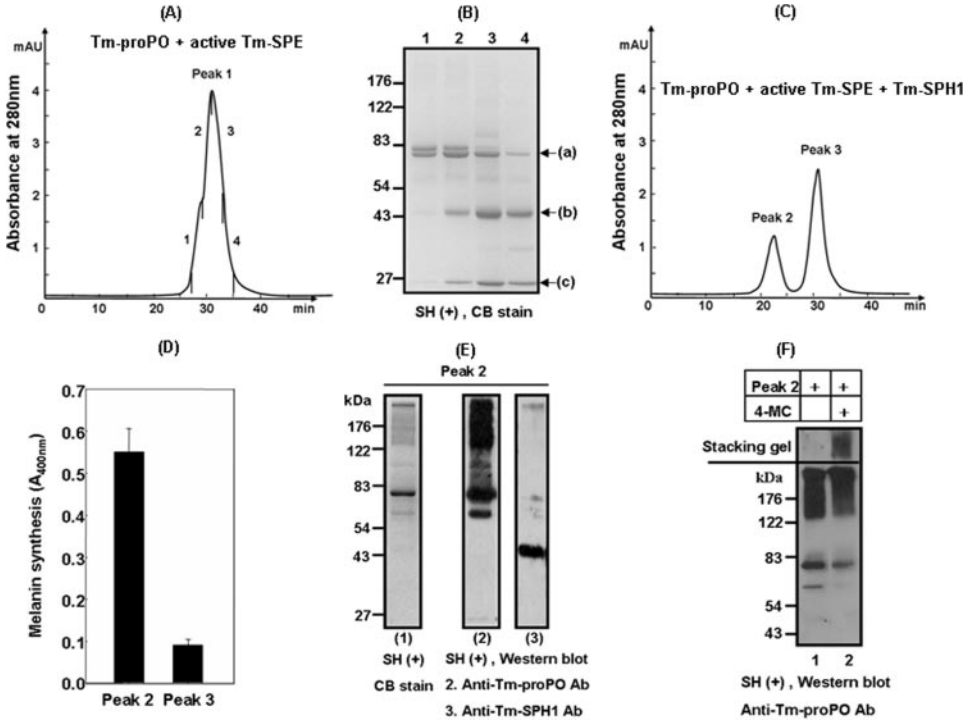

FIGURE 3. **The melanization complex generates chemically cross-linked adducts *in vitro*.** *A*, elution profile of the mixture of Tm-proPO (20 μg) and active Tm-SPE (1 μg) on a Superdex S-200 HR 10/30 size exclusion column (Amersham Biosciences). *B*, SDS-PAGE analysis pattern for each fraction of *A* under reducing conditions. *CB*, Coomassie Brilliant Blue R-250. *C*, elution profile of the mixture of Tm-proPO (20 μg), active Tm-SPE (1 μg), and Tm-SPH1 zymogen (10 μg) on a Superdex S-200 HR 10/30 size exclusion column. *D*, melanin synthesis activities of peaks 2 and 3 in *C*. *E*, SDS-PAGE analysis of peak 2 (*lane 1*) and Western blot analyses of peak 2 using anti-Tm-proPO antibody (*Ab*; *lane 2*) and anti-Tm-SPH1 antibody (*lane 3*). *F*, Western blot analysis of peak 2 using anti-Tm-proPO antibody in the absence (*lane 1*) or presence (*lane 2*) of the PO substrate 4-methylcatechol (*4-MC*).

To identify the protein components involved in melanin synthesis, we performed SDS-PAGE analysis and Western blotting on fractions from peak 2. The high molecular mass band (band e) and the 76-kDa PO band (band a) were observed mainly in peak 2 by SDS-PAGE analysis (Fig. 3*E*, *lane 1*), and both bands were strongly recognized by antibodies against Tm-proPO and Tm-SPH1 (*lanes 2* and *3*). These results demonstrate that the protein complex containing active Tm-PO and active Tm-SPH1 is responsible for melanin synthesis, which is similar to our observations in *Holotrichia* (16). Therefore, we named this protein complex a melanization complex.

Insect PO catalyzes the formation of quinone adducts and other reactive intermediates in melanin synthesis, and these adducts and intermediates help to kill invading microbial pathogens, cause cuticle sclerotization, and promote wound healing (7, 31). To examine whether the melanization complex can increase the production of quinone-mediated cross-linked molecules of high molecular mass in the presence of PO substrate such as 4-methylcatechol compound, we added 4-methylcatechol to the melanization complex and tracked the appearance of the high molecular mass complex by Western blotting with anti-Tm-proPO antibody (Fig. 3*F*, *lane 2*). This result indicates that the quinone generated by the melanization complex leads to the formation of chemically cross-linked molecules of high molecular mass.

Locally Concentrated Melanin on the Surface of Bacteria Shows a Bactericidal Effect—Reactive oxygen intermediates and reactive nitrogen intermediates generated during melanin synthesis have been implicated in insects' ability to kill invading bacteria or parasites (32, 33). To ascertain whether PO-induced melanin can be accumulated on the surface of bacteria and to examine whether this elevated concentration of surface melanin has antibacterial activity, we incubated *S. aureus* or *E. coli* with the melanization complex in the presence of L-dopamine. Strong melanin synthesis was induced on the surface of bacteria (Fig. 4, *B* and *D*), but not when the melanization complex was omitted (*A* and *C*). Furthermore, when we plated melanin-concentrated bacteria on agar plates, the bacteria were unable to grow (Fig. 4*E*, *bars 6* and *12*). Interestingly, some bactericidal activity against *E. coli* was observed when *E. coli* was incubated with Tm-proPO and dopamine or when the bacteria were incubated with Tm-proPO, active Tm-SPE, and dopamine (*bars 3* and *5*). In contrast, the bactericidal activity against *S. aureus* was completely dependent on the melanization complex. These results clearly demonstrate that melanin produced in response to the melanization complex can kill invading bacteria, at least *in vitro*. This is the first biochemical evidence that the melanization complex assembled from purified proteins possesses bactericidal activity.

Active Tm-PO and Active Tm-SPH1 Co-localize on Melanin-enriched Areas of the Surface of Bacteria—To ascertain whether active Tm-PO and active Tm-SPH1 co-localize on melanin-concentrated areas of the surface of bacteria, we performed immunofluorescence localization studies using polyclonal and anti-His monoclonal antibodies against Tm-proPO and Tm-SPH1 (recombinant Tm-SPH1 expressed with an N-terminal His tag), respectively. The secondary antibodies were fluorescein isothiocyanate-conjugated goat anti-mouse antibody for detection of Tm-SPH1 and rhodamine-conjugated goat anti-rabbit antibody for detection of Tm-proPO. The melanin-concentrated bacteria (Fig. 5, *A-s*) and intact bacteria (*A-c*) were fixed on glass coverslips. Tm-SPH1 and Tm-PO were visualized by indirect immunofluorescence due to the fluorescein isothiocyanate- and rhodamine-conjugated antibodies, respectively. Superposition of the fluorescence signals showed that Tm-SPH1 and Tm-PO co-localized on the melanin-enriched areas of the surface of bacteria (Fig. 5, *D-s*). Combined with our results showing cross-linking between the melanization complex and its product, these results suggest that the melanization complex is covalently attached to the bacteria.

Tm-proPO Functions as a Competitive Inhibitor of Melanization Complex Formation—In naïve insect hemolymph, the formation of the melanization complex should be tightly regulated

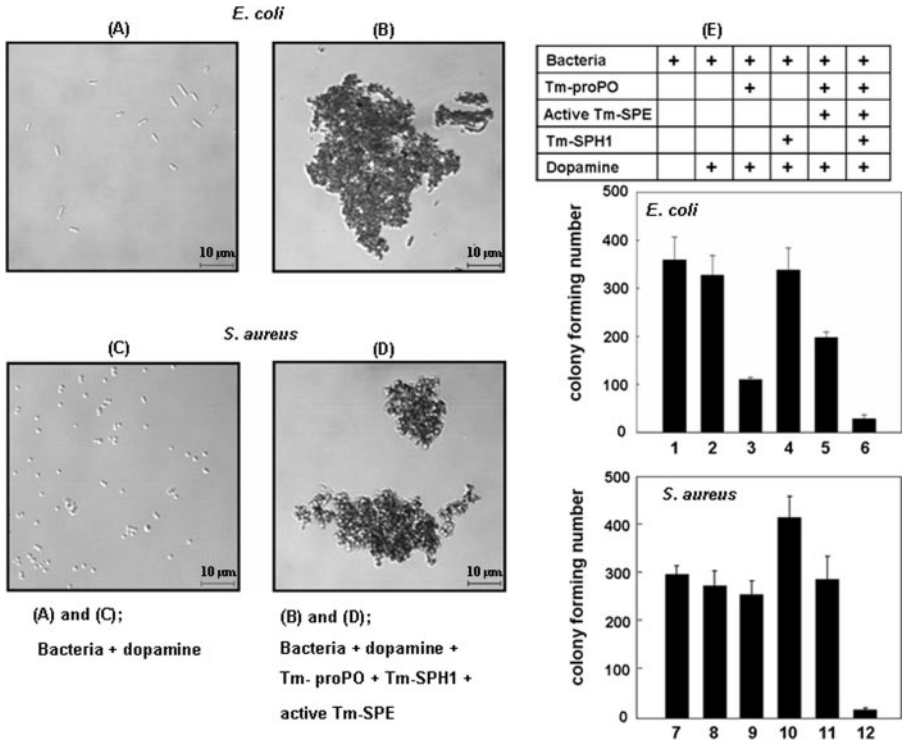

FIGURE 4. **Bacterial agglutination induced by melanization production and the bactericidal effects of melanin on the surface of bacteria.** *A* and *C*, *E. coli* and *S. aureus*, respectively, incubated with dopamine. *B* and *D*, bacterial agglutination induced by the assembly of the melanization complex on the surface of *E. coli* and *S. aureus*, respectively, after incubation of bacteria (10^6 cells) with L-dopamine (1 mM), Tm-proPO (3 μg), Tm-SPH1 zymogen (1 μg), and active Tm-SPE (150 μg). *E*, colony forming numbers of *E. coli* after incubation with the same amounts of proteins described for *B* (*bars 1–6*) and colony forming numbers of *S. aureus* after incubation with the same amounts of proteins described for *D* (*bars 7–12*).

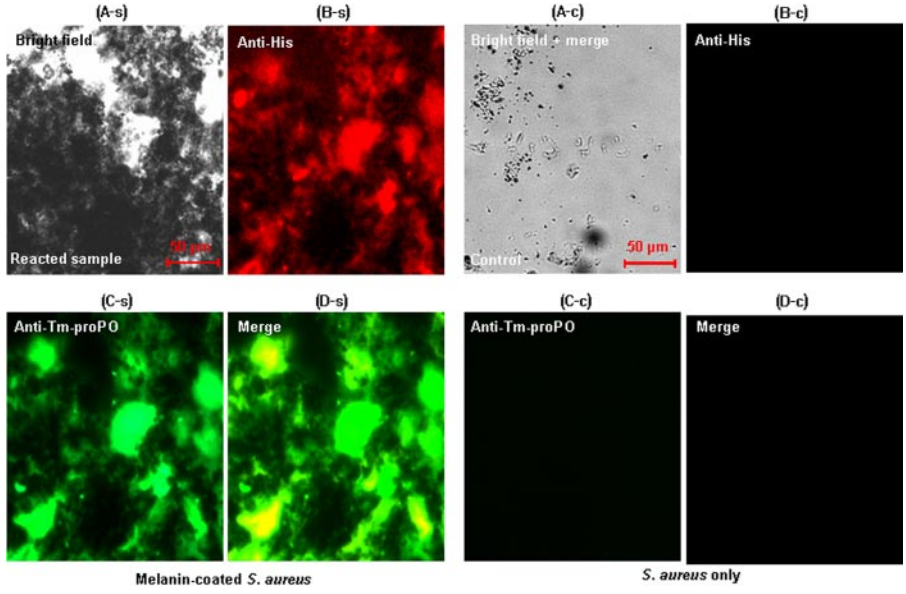

FIGURE 5. **Immunofluorescence microscopy.** *B-s*, *C-s*, and *D-s*, melanin-coated bacteria cells treated with anti-His monoclonal antibody or with anti-Tm-proPO polyclonal antibody and the merged image of *B-s* and *C-s*, respectively. *A-s*, image of a positive control. *B-c* and *C-c*, *S. aureus* by itself, treated with the same antibodies as described for *B-s* and *C-s*. Anti-His and anti-Tm-proPO antibodies were used to detect His-tagged recombinant Tm-SPH1 and active Tm-PO, respectively.

because excessive production of melanin pigment is harmful to insects. This prompts speculation that a competitive inhibitor exists in the hemolymph to prevent the formation of excessive amounts of the melanization complex. We assumed that if active Tm-PO can complex with active Tm-SPH1, then Tm-proPO zymogen, which is present at a high concentration in the hemolymph, may also bind to active Tm-SPH1 to competitively inhibit formation of the melanization complex. If this assumption is correct, melanin synthesis ability should decrease when Tm-proPO is added to preformed melanization complex *in vitro* if newly added Tm-proPO would not be cleaved by existing active Tm-SPE. To block the proteolytic activity of active Tm-SPE and thereby prevent conversion of newly added Tm-proPO to active Tm-PO, benzamidine, a well known serine protease inhibitor, was added to a mixture of the melanization complex and active Tm-SPE. Comparison of the melanin synthesis activity of the benzamidine-treated melanization complex with that of the untreated complex showed no differences (Fig. 6, *bars 2* and *3*), suggesting that benzamidine has no effect on the melanization complex. However, adding Tm-proPO to this benzamidine-treated but still fully functional melanization complex abolished melanin synthesis nearly completely (*bar 4*). This result suggests that Tm-proPO can easily bind active Tm-SPH1 *in vitro*. Because Tm-SPH1 is required for PO-induced melanin synthesis, the addition of new Tm-proPO reduces the number of Tm-SPH1 molecules available to form the melanization complex, resulting in a dramatic decrease in melanization activity. These results strongly support the idea that Tm-proPO works as a competitive inhibitor of melanization complex formation in the naïve hemolymph.

DISCUSSION

The Toll signaling pathway is known to be the most important invertebrate defense system against Gram-positive bacteria and fungi (2, 34, 35). The importance of the melanization remains controversial in at least flies and mosquitoes because successful defense against some pathogens seems to be independent of PO activity in these organisms (9, 10). Nevertheless, the melanization reaction is assumed to be an impor-

Tm-proPO (1μg)	+	+	+	+
Active Tm-SPE		+	+	+
Tm-SPH1		+	+	+
Dopamine	+	+	+	+
Benzamidine			+	+
Tm-proPO (30μg)				+

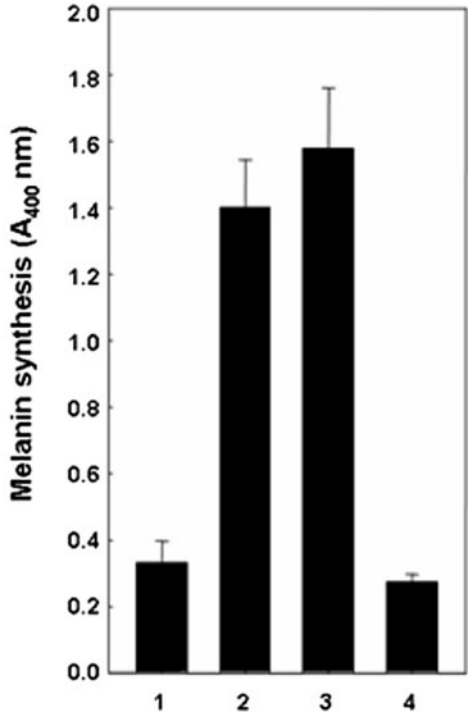

FIGURE 6. **Inhibition of melanin synthesis by newly added Tm-proPO in *in vitro* reconstitution experiments.** After preparation of the melanization complex as described under "Experimental Procedures," the reaction mixtures were cooled on ice for 5 min and divided into three equal portions. One portion was mixed with 200 μl of substrate solution (20 mM Tris-HCl, pH 8.0, containing 1 mM dopamine and 10 mM $CaCl_2$) at 30 °C for 15 min, and the melanin synthesis activity was estimated (*bar 2*). Benzamidine was added to the second portion to a final concentration of 5 mM, and the mixture was incubated at 37 °C for 1 h. Next, substrate solution was added; the mixture was incubated at 30 °C for 15 min; and the melanin synthesis activity was estimated (*bar 3*). Benzamidine was added to the third portion to a final concentration of 5 mM simultaneously with 30 μg of Tm-proPO, and the mixture was incubated at 37 °C for 1 h. Substrate solution was added, and the mixture was incubated at 30 °C for 15 min, after which the melanin synthesis activity was estimated as described above (*bar 4*). One μg of Tm-proPO and dopamine were incubated under the same conditions (*bar 1*), with *bar 2* serving as a negative control.

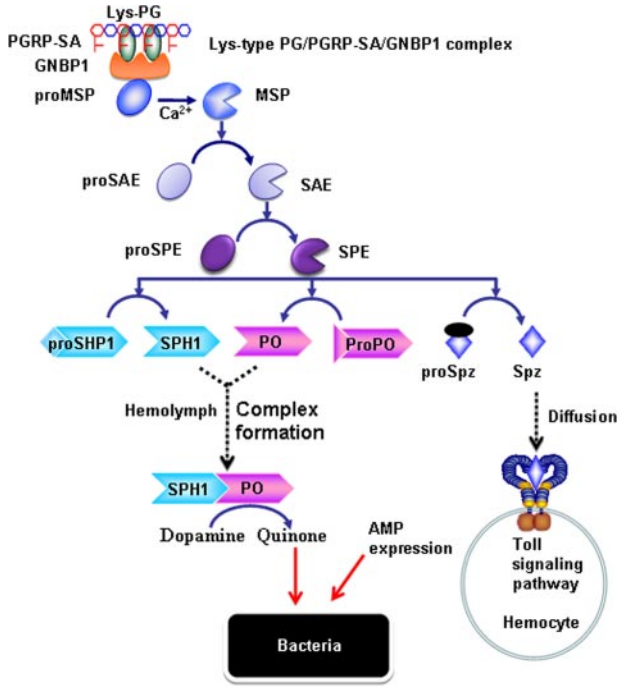

FIGURE 7. **The Toll pathway and the proPO activation cascade rely on a common serine protease for their activation in the larvae of the mealworm *T. molitor*.** When presented to the host immune system, the processed Lys-type PG from Gram-positive bacteria is bound by Tm-PGRP-SA, which then recruits Tm-GNBP1 and Tm-MSP zymogen. In the presence of Ca^{2+}, the PG·Tm-PGRP-SA·Tm-GNBP1 complex induces activation of Tm-MSP zymogen (proMSP). The active form of Tm-MSP activates Tm-SPE-activating enzyme zymogen (proSAE) to activate Tm-SPE-activating enzyme (*SAE*), which subsequently converts Tm-SPE zymogen (proSPE) to activate Tm-SPE protease. Active Tm-SPE cleaves the Spätzle proprotein (proSpz) into processed Spätzle (Spz), leading to the production of antimicrobial peptide (AMP). Also, active Tm-SPE cleaves Tm-proPO and Tm-SPH1, leading to formation of a stable melanization complex. This complex induces local melanin synthesis on the surface of bacteria and enables the insect host to kill the invading microbe.

tant immune response in invertebrates (7, 36). Indeed, PO activity was reported to be redundant in *Drosophila* and *Anopheles gambiae* for the clearance of many bacterial and fungal pathogens. In this study, we have provided biochemical evidence that SPE, described previously as the terminal protease in the Toll signaling pathway (37), is the enzyme that activates both proPO and its cofactor SPH1 in the proPO cascade of the insect *Tenebrio*. The injection of Lys-type PG or Gram-positive bacteria into *Tenebrio* larvae induces both antimicrobial peptides and melanin formation (20). This finding strongly suggests a cooperative (at the very least redundant) relationship between the Toll signaling pathway and the proPO activation cascade in our insect system. The combined results of the present study and our previous work (5) clearly demonstrate that in *Tenebrio*, one unique clip-domain serine protease participates in the regulation of both the Toll signaling pathway and the proPO activation cascade. On the basis of these results, we propose that the PO-dependent melanization reaction and the Toll signaling pathway in *T. molitor* both use Tm-SPE to activate proPO zymogen and pro-Spätzle, respectively, in response to infection with Gram-positive bacteria. In addition, a highly specific non-catalytic SPH regulates melanin production and functions as a regulatory protein for showing antibacterial activity against microbial invaders (Fig. 7). Genetic evidence for the molecular cross-talk between *Drosophila* Toll activation and the melanization reaction was also provided by using *Drosophila* Serpin27A mutant fly (38), demonstrating that the melanization reaction requires Toll pathway activation and depends on the removal of Serpin27A.

Recently, several groups, including our own, have demonstrated that the SPHs bind tightly to microbial cell wall components (23, 27) or pathogenic bacteria (39). We found that Tm-SPH1 specifically binds to curdlan polymers (β-1,3-glucan fungal polymer) (27). These observations may explain why Tm-SPH1 is required for melanin synthesis. When Gram-positive

bacteria entry the invertebrate body, the host should recognize these microbes and subsequently kill them. To accomplish this process, SPH zymogen in the hemolymph may first localize and adhere to the invading bacteria; at the same time, microbial cell wall components induce the production of the active forms of Tm-MSP and Tm-SPE-activating enzyme, which participate in a serine protease cascade to activate Tm-SPE zymogen in the hemolymph. Active Tm-SPE cleaves Tm-proPO and Tm-SPH1 zymogen to form active Tm-PO and Tm-SPH1, respectively, resulting in formation of the melanization complex in the hemolymph. This probably serves to ensure that active PO can complex with the active form of Tm-SPH1 and that the active enzyme, Tm-PO, does not diffuse through the hemolymph in an uncontrolled manner but rather stays in the vicinity of invading microorganisms. However, the melanization complex is easily replaced with Tm-proPO, which exists at a high concentration in the naïve hemolymph. If excess Tm-proPO is incorporated into the melanization complex, melanin synthesis decreases because the specific active Tm-SPH1 is absorbed by Tm-proPO, causing the remaining active Tm-PO to lose its melanization activity. This would constitute an important regulatory mechanism to control the activity of active PO and the melanization complex to prevent production of reactive quinone derivatives in inappropriate places. Finally, the melanization complex is immobilized via reactive quinone adducts on bacteria and kill the invading microorganisms using generated cytotoxic molecules such as superoxides and hydroxyl radicals.

In summary, our biochemical studies shed further light on how the Lys-type PG recognition signal leads to activation of proPO and ultimately to melanin synthesis. Our work supports a model in which melanin synthesis is triggered by sequential activation of three serine protease zymogens. As a result of this three-step proteolytic cascade, proPO and SPH1 zymogen are processed and form the active melanization complex on the surface of bacteria. The presence of these complexes on the bacterial surface allows the invading microbes to be immobilized and destroyed by innate immune responses.

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