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Phenoloxidase Is an Important Component of the Defense against *Aeromonas hydrophila* Infection in a Crustacean, *Pacifastacus leniusculus*[*]

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The melanization cascade, in which phenoloxidase is the terminal enzyme, appears to play a key role in recognition of and defense against microbial infections in invertebrates. Here, we show that phenoloxidase activity and melanization are important for the immune defense toward a highly pathogenic bacterium, *Aeromonas hydrophila*, in the freshwater crayfish, *Pacifastacus leniusculus*. RNA interference-mediated depletion of crayfish prophenoloxidase leads to increased bacterial growth, lower phagocytosis, lower phenoloxidase activity, lower nodule formation, and higher mortality when infected with this bacterium. In contrast, if RNA interference of pacifastin, an inhibitor of the crayfish prophenoloxidase activation cascade, is performed, it results in lower bacterial growth, increased phagocytosis, increased nodule formation, higher phenoloxidase activity, and delayed mortality. Our data therefore suggest that phenoloxidase is required in crayfish defense against an infection by *A. hydrophila*, a highly virulent and pathogenic bacterium to crayfish.

Melanization is an easily observed defense reaction in invertebrates that is initiated by a proteolytic cascade that terminates with cleavage of prophenoloxidase (proPO)[2] to phenoloxidase (PO), an enzyme that can generate melanin (1–3). Genetic studies show that melanization and anti-microbial defense share common components and mechanisms in which a positive correlation between the melanization of injected beads and antibacterial responses seems to be due to shared genes and pathways that regulate these two effector mechanisms (4). In *Drosophila melanogaster*, melanization plays a key role against infection by parasitoid wasps (5), and it acts in augmenting the effectiveness of other immune reactions promoting resistance of *Drosophila* to microbial infection (6, 7). Studies with mutants with impaired melanization capacity suggest that if the melanization reaction is inhibited a higher susceptibility to microbial infection occurs (8, 9). A mutant with a defective proPO-activating system produced less melanotic capsules around eggs of the parasitoid *Leptopilina boulardi* in *D. melanogaster* (10). In mosquitoes, several transcripts encoding enzymes involved in melanization are induced by a *Plasmodium* parasite in the midgut of *Anopheles gambiae* (11), and the resulting induced melanization could kill the ookinetes directly in susceptible mosquitoes and dispose of dead parasites in refractory mosquitoes (12). Phenoloxidase has also been demonstrated to interfere with microbial infection in several insect species (13–23). In some cases melanization, or rather the activity of the enzyme PO, was found to have no or little impact on the killing/clearance of parasites (24, 25) and the melanization reaction was found not to be required for survival of mosquitoes/flies after certain microbial infections (26, 27).

However, several studies have shown that the melanization of bacteria is a critical defensive process in invertebrates that appears to be facilitated by or is associated with phagocytosis (20, 28–31). In the freshwater crayfish, *Pacifastacus leniusculus*, the expression of PO has been shown to be associated with an increased resistance to the crayfish pathogen *Aphanomyces astaci* (32).

In our preliminary studies we observed that the mRNA transcripts of crayfish proPO gene and pacifastin gene (a proteinase inhibitor specific against the PO-activating enzyme of crayfish) (33) were up-regulated upon a challenge with *Aeromonas hydrophila*, a highly pathogenic bacterium isolated from freshwater crayfish, suggesting that proPO and pacifastin genes may be involved in defense. We, therefore, performed gene silencing of the proPO or the pacifastin gene separately using double-stranded RNA (dsRNA)-mediated RNA interference (RNAi). Consequently, knock down of the proPO gene should result in lower PO activity and an enhanced PO activity can be reached by silencing the pacifastin gene. The RNAi experiments were then designed to study the correlation between PO activity and the effect of *A. hydrophila* infection in crayfish. In addition, we assayed to what extent *in vivo* phagocytosis of this pathogen was affected by manipulating PO activity levels.

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[2] The abbreviations used are: proPO, prophenoloxidase; PO, phenoloxidase; dsRNA, double-stranded RNA; RNAi, RNA interference; RT-PCR, reverse transcription PCR; GFP, green fluorescent protein; CFU, colony-forming unit; FITC, fluorescein isothiocyanate.

MATERIALS AND METHODS

Animals and Bacterial Strain—Freshwater crayfish, *P. leniusculus*, purchased from Lake Vättern, Sweden, were kept in aquaria in aerated tap water at 10 °C. Only intermolt and healthy animals were used for experiments. The crayfish-pathogenic Gram-negative bacterium, *A. hydrophila*, was prepared as previously described (34). The dose of *A. hydrophila* (200 μl of 4×10^6 CFU/ml) resulting in 80% mortality of control crayfish (15 ± 2 g) within 50 h at 20 ± 2 °C was determined and used throughout this study.

ProPO and Pacifastin mRNA Expression after A. hydrophila Infection in Crayfish—To determine whether the proPO system was involved in the immune responses toward a bacterial infection, the animals were challenged with *A. hydrophila*. For bacterial challenge, 200 μl of *A. hydrophila* (4×10^6 CFU/ml) in crayfish saline buffer (CFS) (0.2 M NaCl, 5.4 mM KCl, 10 mM $CaCl_2$, 2.6 mM $MgCl_2$, 2 mM $NaHCO_3$, pH 6.8) was injected via the base of the fourth walking leg, and the crayfish were kept at room temperature for 3 h followed by total RNA isolation for cDNA preparation from the total hemocytes. CFS injection was done as a control treatment. Hemocyte total RNA was extracted using GenElute™ Mammalian Total RNA Miniprep kit (Sigma) followed by RNase-free DNase I (Ambion, Austin, TX) treatment. The presence of proPO or pacifastin gene transcripts was determined by reverse transcription (RT)-PCR. Complementary DNA was synthesized with ThermoScript (Invitrogen), and PCRs were performed with crayfish proPO (GenBank™ accession code X83493)-specific primers (1488+, 5′-TGCGCATTACCCATCTCGAC-3′; 2051−, 5′-ACTTCAGCATCTTGCAGGCG-3′) and pacifastin gene light chain (GenBank™ accession code U81825)-specific primers (433+, 5′-TGCACCAAGAGGCTTTGTCG-3′; 968−, 5′-TTGGAGCCATCAGTACACACAGC-3′). Crayfish 40S ribosomal protein 16S gene (GenBank™ accession code CF542417) primers (5+, 5′-CCAGGACCCCCAAACTTCTTAG-3′; 364−, 5′-GAAAACTGCCACAGCCGTTG-3′) was employed in all PCR experiments as internal controls. The PCR program was as follows: 94 °C, 3 min, followed by 25 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 40 s for proPO and pacifastin genes, and 29 cycles for the 40S ribosomal protein 16S gene, respectively. The PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide.

Generation of dsRNA—Oligonucleotide primers with T7 promoter sequences (italic) at the 5′-ends were synthesized to amplify a 692-bp and 550-bp region of the *P. leniusculus* proPO gene and pacifastin light chain, the inhibitory subunit, gene (33), respectively, from the crayfish hemocyte cDNA. The primer sequences were: 871+, 5′-*TAATACGACTCACTATAGGG*AAGCTCACCGCTAACAACTCCG-3′ and 1563−, 5′-*TAATACGACTCACTATAGGG*TGACTTCGGCTTCCTGTGCT-3′ for proPO gene; 477+, 5′-*TAATACGACTCACTATAGGG*TGCCAGTCAGACAGAGGAAACG-3′ and 1026−, 5′-*TAATACGACTCACTATAGGG*TTCAGTGCAAGAAGCGGAGC-3′ for pacifastin gene inhibitory domain, respectively. Control 657-bp templates were produced by PCR using primers specific to one part of the green fluorescent protein (GFP) gene from the pd2EGFP-1 vector (Clontech, Palo Alto, CA), and the primers containing the T7 promoter sequences (italic) were as follows: 63+, 5′-*TAATACGACTCACTATAGGG*CGACGTAAACGGCCACAAGT-3′; 719−, 5′-*TAATACGACTCACTATAGGG*TTCTTGTACAGCTCGTCCATGC-3′. To generate dsRNA, PCR products purified by gel extraction (Qiagen, Hilden, Germany) were used as templates for *in vitro* transcription using the MegaScript kit (Ambion), and dsRNA was purified with the TRIzol LS reagent (Invitrogen) method.

dsRNAi in Vivo—Small intermolt crayfish (15 ± 2 g, fresh weight) were used for *in vivo* RNAi experiments. Shortly, four drops of hemolymph were withdrawn from the crayfish for isolation of hemocyte total RNA 1 day before the dsRNA injection. Then, 150 μg of proPO, pacifastin, or GFP dsRNA dissolved in CFS (200 μl) was injected via the base of the fourth walking leg. The injection was repeated after 24 h, and isolation of total RNA from hemocytes for quantitative RT-PCR was performed 48 h after the first dsRNA injection. The PCR templates were prepared as mentioned above. The detection and comparative quantification of proPO or pacifastin gene transcripts after dsRNA injection were done by quantitative RT-PCR to determine gene-silencing efficiency using the QuantiTect SYBR Green PCR kit (Qiagen). The expression of the proPO or pacifastin gene was normalized to the expression of the mRNA encoding the crayfish 40S ribosomal protein 16S gene for each sample. The primers used were as follows: proPO 1496+, 5′-ACCCATCTCGACCATGCAC-3′, and 1637−, 5′-AGACGCTGCTCCATGAAGC-3′; pacifastin 378+, 5′-CAGGTGGAAGGCAGATGATTG-3′, and 518−, 5′-TCAGGATCTCCATAGCACTCCG-3′; crayfish 40S ribosomal protein 16S gene, 156+, 5′-CTCTTTCTTGGAGGCTTCATCC-3′ and 280−, 5′-CAATTCGCGTTCGTGTGAAG-3′. SYBR Green quantitative RT-PCR amplification was performed in a Rotor-Gene 3000 (Corbett Robotic). The hemocyte cDNA was prepared using oligo(dT) as described above. The cDNA samples were diluted 1:10 with RNase-free sterilized water. The amplification was carried out in a 25-μl reaction volume that contained 12.5 μl of 2× QuantiTect SYBR Green PCR master mix, 0.4-μM concentrations of each primer, and 5 μl of diluted cDNA template. RNase-free distilled water was filled to reach a total volume of 25 μl/reaction. All runs employed a negative control without target DNA. Thermal cycling conditions were as follows: 95 °C for 15 min, followed by 45 cycles of 94 °C for 15 s, 62 °C for 30 s, and 72 °C for 30 s. All PCRs were performed in triplicates.

Hemolymph PO Activity in Crayfish after Injection of proPO dsRNA or Pacifastin dsRNA—Crayfish hemolymph was collected both pre-dsRNA injection (1 day before the dsRNA injection) and 48 h after the first proPO dsRNA, pacifastin dsRNA, or GFP dsRNA injection. The detection of PO activity in hemolymph sample was performed by measurement of L-3,4-dihydroxyphenylalanine (L-dopa) conversion to dopachrome at 490 nm. Hemolymph (150 μl) was taken and distributed into 24-well plates (Sarstedt) followed by adding 65 μl of L-dopa (3 g/liter in water) (Sigma) and 285 μl of Tris·HCl (10 mM, pH 8.0). Control samples were prepared using double-distilled water instead of crayfish hemolymph. Then, the mixed samples were incubated for 30 min at room temperature, and the 500 μl of distilled water was added, followed by monitoring

the absorbance at 490 nm. Protein content was determined using a Bradford assay, and PO activity was recorded as ΔA490/mg total protein. Data gathered from at least three experiments were used for statistical analysis.

Bacteria Count in proPO or Pacifastin Down-regulated Crayfish—The proPO dsRNA, pacifastin dsRNA, or GFP dsRNA injections were performed as described above, followed by *A. hydrophila* challenge at 48 h after the first dsRNA injection. The bacteria count was carried out in hemolymph collected 3 h after bacterial inoculation. The serial diluted hemolymph was dotted onto the Luria Bertani-agar plate (10 μl/dot) and then incubated at 30 °C overnight followed by counting of bacterial CFUs.

Cumulative Mortality Assay of A. hydrophila Challenge in proPO or Pacifastin Knocked-down Crayfish—ProPO dsRNA, pacifastin dsRNA, or GFP dsRNA injections were performed as described above. The bacterial strain used was *A. hydrophila*. Bacterial challenges were performed at 48 h after the first dsRNA injection as described above. The cumulative mortality was recorded per hour, and the data were analyzed by Student's *t* test.

Phagocytosis Assay—Fluorescein isothiocyanate (FITC)-conjugated heat-killed *A. hydrophila* was prepared using a modified method previously described by Hed (35). Briefly, heat-killed *A. hydrophila* was washed six times in 0.9% NaCl at 1200 × *g* for 10 min. The bacteria were then incubated at a concentration of 10^9 particles/ml in 0.1 M Na_2CO_3, pH 9.5, containing 0.1 mg/ml FITC (Sigma) for 30 min at 37 °C. The bacteria were washed five times in 0.9% NaCl at 1200 × *g* for 10 min, resuspended at a concentration of 10^8 particles/ml in CFS, and stored at −20 °C until use. The dsRNA injections were performed as described above, and 200 μl of FITC-conjugated heat-killed *A. hydrophila* (4×10^6 particles/ml in CFS) was injected into the animals via the base of the fourth walking leg 48 h after the first dsRNA injection. Hemocytes were withdrawn for phagocytosis detection at 3 h after the injection of FITC-conjugated bacteria. The total hemocytes were separated by centrifugation and attached onto glass slides as previously described (36). The fluorescence of the adhering FITC-conjugated bacteria was quenched by adding a few drops of 0.4% trypan blue (Sigma) for several minutes and then replaced with 0.15 M NaCl. The ingested bacteria were easily detected under the UV light microscope. The percentage of phagocytosing cells was determined by counting at least 500 live cells on each slide and dividing the number of cells with ingested fluorescent bacteria with the total number of counted cells.

RESULTS

Our results show that the proPO and pacifastin mRNA transcripts were up-regulated 3 h after challenge with *A. hydrophila* in crayfish (Fig. 1), whereas mRNA for the proPO-activating enzyme (37) or the lipopolysaccharide- and β-1,3-glucan-binding protein (38) (data not shown), two other important components involved in the crayfish proPO-system (1), were unaffected. To elucidate the role of phenoloxidase in the host defense against *A. hydrophila*, dsRNA was employed to target the proPO gene in crayfish. The efficiency of RNAi-mediated transcript depletion was determined by quantitative RT-PCR,

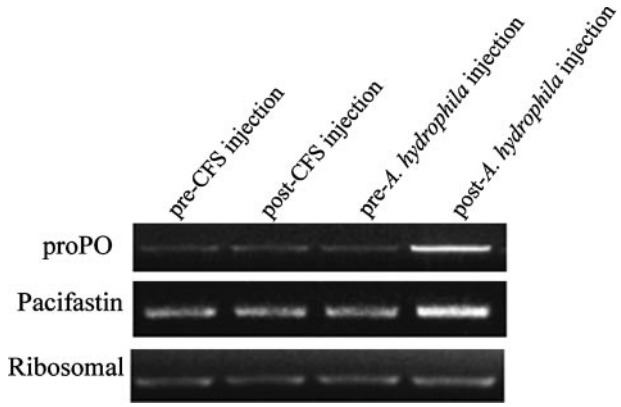

FIGURE 1. **Up-regulation of proPO and pacifastin mRNA expression 3 h post-challenge with *A. hydrophila*.** For bacterial challenge, 200 μl of *A. hydrophila* (4×10^6 CFU/ml) in crayfish saline buffer was injected. The isolation of hemocyte total RNA for RT-PCR was performed at 3 h after bacterial infection. Compared with CFS treatment, proPO and pacifastin mRNA transcripts were up-regulated post-challenge with *A. hydrophila*. This experiment was repeated twice.

and this showed that the proPO mRNA transcript was reduced to ~57%. No obvious difference in the amount of proPO transcript was observed between pre- and post-GFP dsRNA-treated animals (Fig. 2*A*). Because PO is produced by the hemocytes and is released into the hemolymph (1), we also determined whether silencing of the proPO gene would result in reduced PO activity in crayfish hemolymph. The level of PO activity was decreased by 33% in proPO-silenced animals, and in contrast, there was no apparent change of PO activity in the GFP dsRNA-treated animals (Fig. 3). The PO-silenced animals were then challenged with *A. hydrophila* to test the effect on crayfish survival. Knock down of PO decreased the time 4.8-fold from 48 to 10 h to reach 80% of cumulative mortality, and this decrease was significant (Student's *t* test, $p < 0.01$, Fig. 5). In contrast, no significant difference of mortality was observed between GFP dsRNA and CFS-injected animals followed by an infection with *A. hydrophila* (data not shown). This result suggests that the effect of dsRNA injection on crayfish mortality is gene-specific, and hence the reduction of PO activity could result in a faster and higher mortality following a bacterial challenge than that in control animals. We also performed gene silencing of proPO to determine whether PO activity influences the growth of *A. hydrophila*, and therefore crayfish were injected with proPO dsRNA followed 48 h later by challenging with *A. hydrophila*. In parallel to measure PO activity in all proPO-silenced animals, we also determined the presence and quantity of *A. hydrophila* in the hemolymph of the same infected animals. Knock down of proPO strongly increased the number of growing bacteria, 2.3-fold, in proPO-depleted animals (Fig. 4), indicating that proPO may act in a bacterium-killing pathway. In summary, pretreating animals with proPO dsRNA resulted in lower proPO transcription, decreased PO activity, significantly faster cumulative mortality, and higher number of bacteria in hemolymph following a challenge with *A. hydrophila*.

It appears that the animal responds to an infection by increasing PO activity. A more detailed study showed that after silencing the inhibitory domain of the pacifastin gene (44% reduction in mRNA transcription level, Fig. 2*B*) the PO activity was enhanced 1.4-fold above levels of the control animals

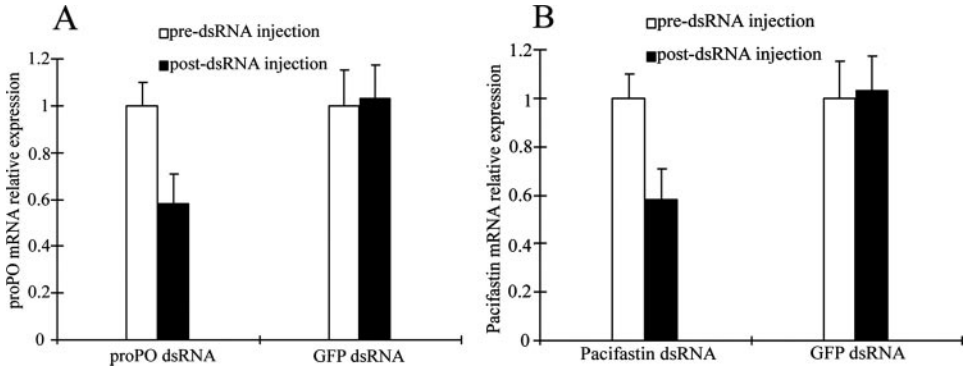

FIGURE 2. **RNA interference-mediated silencing of proPO gene and pacifastin gene *in vivo*.** Total hemocyte RNA was isolated 1 day before the dsRNA injection. RNAi experiments were then carried out with injection of proPO dsRNA, pacifastin dsRNA, or GFP dsRNA. The animals were injected twice with dsRNA before the second isolation of hemocyte total RNA at 48 h after the first dsRNA injection. The results show that proPO or pacifastin gene expression is suppressed by prior injection of proPO dsRNA (*A*) or pacifastin dsRNA (*B*), respectively. In contrast, the GFP control dsRNA injection has no effect on proPO or pacifastin gene transcription. The crayfish 40S ribosomal protein 16S gene was used as an internal control for relative proPO or pacifastin gene quantification by quantitative RT-PCR. The experiment has been repeated twice, and the data represent means of duplicates. *Bars* indicate mean ± S.E. ($n = 2$).

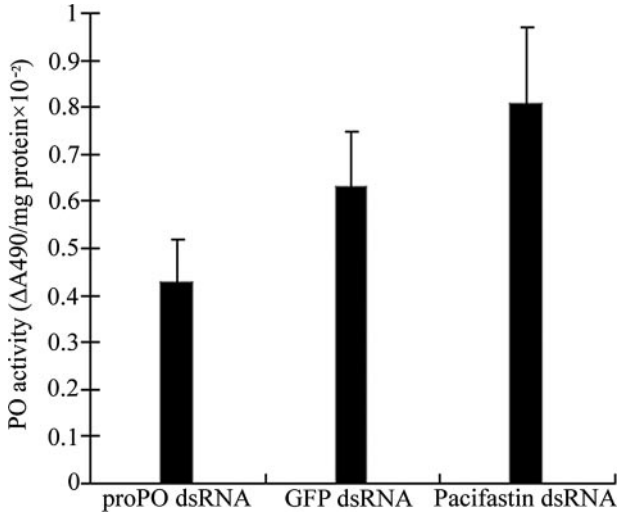

FIGURE 3. **Hemolymph phenoloxidase activity in proPO- or pacifastin-depleted crayfish.** Crayfish hemolymph was collected both at pre-dsRNA injection and 48 h after the first dsRNA injection. The PO activity was measured using L-dopa and defined as ΔA490/mg protein. These experiments have been repeated three times, and the data represent means of triplicates. *Bars* indicate mean ± S.E. ($n = 3$).

(Fig. 3). We then tested whether this knock down had any effect on host resistance toward *A. hydrophila*. In the microbial challenge experiments there was a significant increase in the mean survival time ($p < 0.01$, Student's t test) by this treatment. The accumulated mortality reached 80% within 48 h in the control group, whereas the same mortality was reached 64 h after infection in the animals with an enhanced PO activity (Fig. 5). Further, the higher PO activity was accompanied by a strong decrease of bacterial CFUs (40% reduction, Fig. 4), indicating that the enhanced PO activity was correlated with both an increased survival time and enhanced bacterial clearance.

We also tried to elucidate the correlation between PO activity and phagocytosis by monitoring the change of the phagocytic rate if the PO activity were decreased or increased by dsRNA silencing. Because the PO-silenced animals always died earlier (from 5 h after infection) after they were challenged with *A. hydrophila*, we determined the phagocytic rate of the total hemocytes in RNAi-treated animals and compared it with that of control RNAi-treated crayfish. Compared with GFP dsRNA-treated animals (2.1%), decreased PO activity in the hemolymph gave a significant 57% decrease of the phagocytic activity (0.9% of phagocytic bacteria, Student's t test, $p < 0.01$, Fig. 6), whereas an increase in phagocytosis was observed in the pacifastin gene-silenced animals (3.7%, Student's t test, $p < 0.05$, Fig. 6). Pretreatment of animals with GFP dsRNA, on the other hand, resulted in a rate of uptake of 2.1%, which is similar to CFS-treated animals (2.0%), suggesting that no obvious effect on phagocytosis was produced by nonspecific dsRNA treatment (data not shown). Moreover, nodule formation was quite often seen in PO activity-enhanced, but to a lesser extent in proPO-silenced, animals during our experiments (Fig. 7). Thus, it is likely that the phagocytic and nodule-forming activities in freshwater crayfish are associated with PO activity. Taken together, we assume that PO participates not only in the melanization of the parasites but also somehow enhances other cellular activities such as phagocytosis and/or nodule formation.

DISCUSSION

Our study provides strong direct evidence to support the conclusion that the capacity for production of PO activity is an important component for an increased resistance against an infection with *A. hydrophila* in a crustacean. It is probably important while evaluating these results to keep in mind that *A. hydrophila* is a highly pathogenic bacterium to this crayfish. In contrast to other bacteria, the injection of a small number (100–500-fold less than *Escherichia coli*, for example) will suffice to kill the crayfish. One may speculate that when dealing with a pathogen capable of quickly establishing itself in the host, an immediate response by the cellular defense augmented by the melanization reaction is crucial. Other defense responses, *e.g.* AMPs, may be of greater significance when dealing with large numbers of less pathogenic bacteria capable of overcrowding the cellular defense. This conclusion is also strengthened by our previous results of repression of the crayfish plague *Aphanomyces astaci* growth *in vivo* in freshwater crayfish *Astacus astacus* (32), retardation of mycelial growth *in vitro*, and inhibition of extracellular proteinases from this special crayfish parasite by melanin and several intermediates involved in the proPO system (39). In line with these results, a recent study demonstrates that PO inhibition leads to increased host susceptibility (40). Similar results, that the predominant lytic loss of parasites may result from overproduction and circulation of toxic byproducts during melanin synthesis (18), are described in mosquitos lacking the serpin SRPN2. So far, all invertebrate POs identified in detail are synthesized as inactive precursors

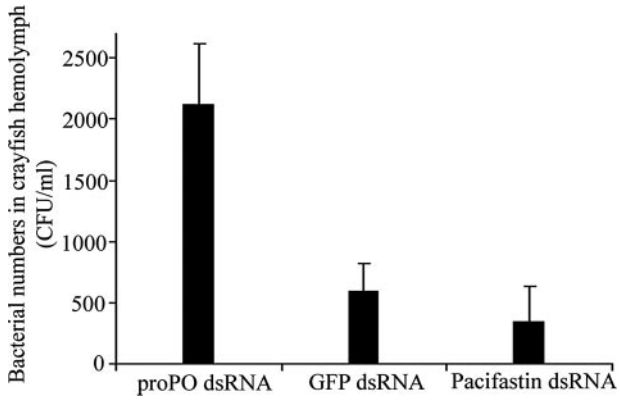

FIGURE 4. **Bacterial numbers in proPO- or pacifastin-depleted crayfish.** The dsRNA injections were done as described above followed by challenging of 200 μl of *A. hydrophila* (4×10^6 CFU/ml in CFS) at 48 h after the first dsRNA injection. The bacterial CFUs in crayfish hemolymph were determined as CFU/ml of crayfish hemolymph at 3 h after the bacterial challenge. This experiment was repeated three times, and the data represent means of triplicates. *Bars* indicate mean ± S.E. ($n = 3$).

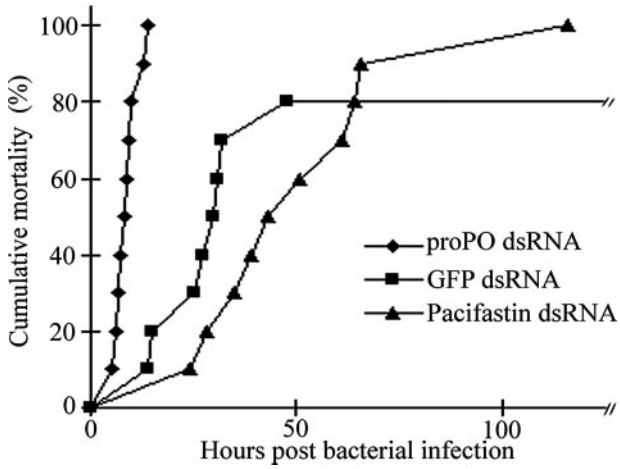

FIGURE 5. **Cumulative mortality of crayfish challenged with *A. hydrophila* after dsRNA silencing.** Crayfish were injected twice with different dsRNA, followed by challenge of *A. hydrophila* as described above. Ten animals were used for each group. The mortality was recorded hourly. The cumulative mortality of crayfish in which proPO or pacifastin was silenced was compared with that of GFP control dsRNA-treated animals. This experiment was repeated four times. The data were analyzed by Student's *t* test ($p < 0.01$).

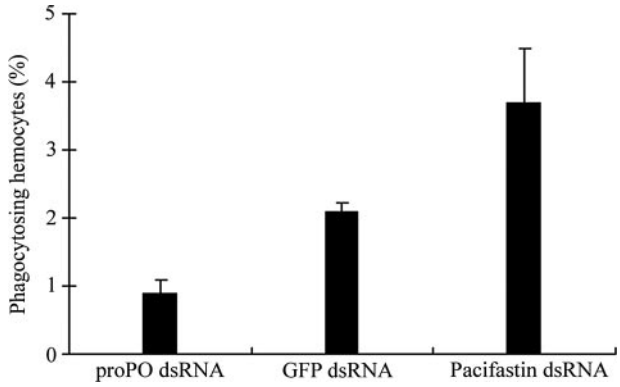

FIGURE 6. **The percentage of crayfish hemocytes ingesting FITC-conjugated heat-killed *A. hydrophila*.** The RNAi and bacterial challenges were performed as previously described. The phagocytosing assay was carried out at 3 h after the injection of FITC-conjugated heat-killed *A. hydrophila*. The percentage of phagocytosing cells was determined by dividing the number of cells with ingested fluorescent particles with total number of counted cells. The data were analyzed by Student's *t* test ($p < 0.01$ for proPO gene-depleted animals and GFP control treatments; $p < 0.05$ for pacifastin gene-depleted animals and GFP control treatments). This experiment was repeated four times. *Bars* indicate mean ± S.E. ($n = 4$).

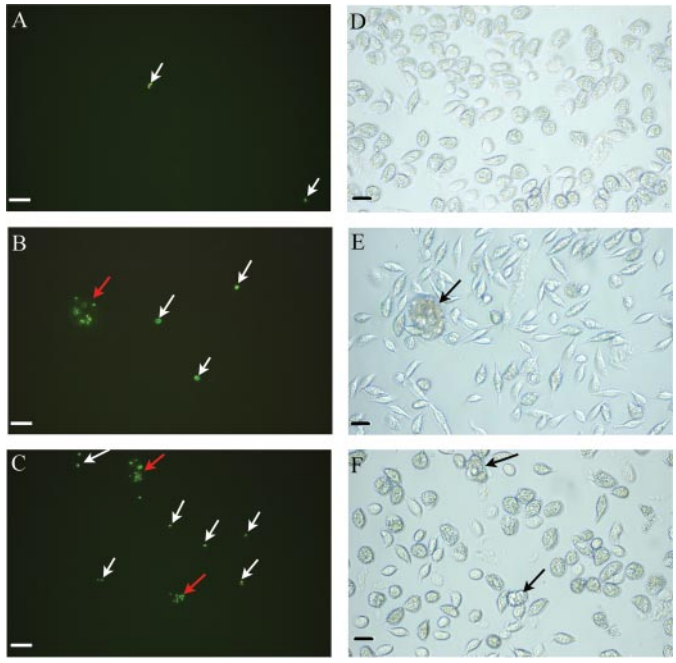

FIGURE 7. **Phagocytosis and nodule formation of FITC-conjugated heat-killed *A. hydrophila* by crayfish hemocytes.** Phagocytosis was determined at 3 h after the injection of FITC- conjugated heat-killed *A. hydrophila* in different dsRNA-treated animals. *White arrows* show the ingested FITC-conjugated heat-killed *A. hydrophila* particles by crayfish hemocytes under UV light microscopy. *Red arrows* show nodules that contain several ingested bacterial particles under UV light microscopy. *Black arrows* show nodule formation under normal light microscopy. *A* and *D*, hemocytes of proPO gene-depleted animals. *B* and *E*, hemocytes of GFP dsRNA-treated animals. *C* and *F*, hemocytes of pacifastin gene-depleted animals. *A–C*, UV light microscopy; *D–F*, normal light microscopy. This experiment was repeated four times. *Bars*, 20 μm.

that are activated by a limited proteolysis. One should bear in mind that the activation of such serine protease cascades must be tightly controlled. Serine proteinase inhibitors are, henceforth, employed to regulate this activation process by preventing excessive activation that is often fatal to hosts (33, 41, 42). In *Drosophila,* the *spn27A*^{1}, a mutant lacking spn27A, shows excessive melanization. This mutant is more susceptible to infection by *Beauveria bassiana* (43). The Spn27A^{ex32}, another protein null mutant for Spn27A homozygous or hemizygous progeny that developed spontaneous melanization, results in a higher rate of lethality at mid-pupal stages (44). These studies indicate that a tight control of melanization is required for a proper antifungal response or development in flies. In crayfish, we speculate that endogenous proteinase inhibitors could, perhaps, be employed to protect certain tissues or cells by inhibiting unnecessary production of highly toxic and reactive compounds as well. If pacifastin is silenced, such protective roles could be affected, which may explain that the mortality always reaches 100% after some time in pacifastin-silenced animals.

Proteins associated with the proPO-activating system such as, for example, peroxinectin are known to mediate cell communication, opsonization, and cell adhesion (1). Cell adhesion is the first step in many cellular responses, including hemocyte

spreading, phagocytosis, nodule formation, encapsulation, and hemocyte aggregation. Peroxinectin, an active adhesion molecule from crayfish, is an efficient mediator or promoter of adhesion of semigranular and granular hemocytes (45), encapsulation (46), and phagocytosis (47). Recently, the extracellular processing of properoxinectin into active peroxinectin was shown to involve proteolytic steps shared with the proPO-activating system to generate the catalytically active PO (48). Therefore, elevated activity of proPO-activating enzyme caused by silencing of pacifastin will probably increase the activation of peroxinectin, leading to higher hemocyte cell adhesion activity and thus a higher phagocytic rate in crayfish. However, the detailed correlations between peroxinectin activity and the proPO system in the activation of phagocytosis still remain to be elucidated.

In conclusion, this study clearly shows that RNAi of proPO leads to increased bacterial growth and a significantly increased mortality rate following a bacterial challenge. In contrast, RNAi of pacifastin results in increased survival time and enhanced bacterial clearance. These data together strongly suggest that phenoloxidase is necessary for crayfish immune defense against a pathogenic bacterial infection.

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