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Broad-spectrum antimicrobial activity of the reactive compounds generated *in vitro* by *Manduca sexta* phenoloxidase

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Abstract

Although quinone production and melanin formation are widely recognized as an integral part of the insect defense system, experimental evidence is lacking that the proteolytic activation of prophenoloxidase participates in the direct killing of invading microbes—active phenoloxidase generates quinones that polymerize to form melanin. Here, we report the antimicrobial effect of reactive intermediates produced in phenoloxidase-catalyzed reactions. After being treated with *Manduca sexta* phenoloxidase and dopamine, *Escherichia coli* and *Bacillus subtilis* ceased to grow, whereas the growth of *Pichia pastoris* was slightly affected. Microscopic analysis showed melanin deposition on cell surface, aggregation of bacteria, and loss of cell mobility. Viability tests revealed major decreases in the bacterial colony counts and, since the decrease remained significant after dispersion of the cell clumps, the reactive compounds were surmised to have aggregated and killed *E. coli* and *B. subtilis* cells. Under the experimental conditions, 60–94% of the Gram-negative bacteria (*E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*) and 52–99% of the Gram-positive bacteria (*Bacillus cereus*, *B. subtilis*, *Micrococcus luteus*, and *Staphylococcus aureus*) were killed. In the presence of phenoloxidase, dopamine or 5,6-dihydroxyindole (DHI) exhibited much higher antibacterial activity than *L*-dopa, *N*-acetyldopamine (NADA) or *N*- β -alanyldopamine (NBAD) did, suggesting that DHI and its oxidation products were cytotoxic. The antifungal activity of DHI was detected using *P. pastoris*, *Saccharomyces cerevisiae*, *Candida albicans*, and *Beauveria bassiana*. These results established that prophenoloxidase activation is an integral component of the insect defense system involving a multitude of enzymes (e.g. proteinases, oxidases, and dopachrome conversion enzyme (DCE)), which immobilizes and kills invading microorganisms.

Keywords

Insect immunity; Prophenoloxidase activation; Melanization; 5,6-dihydroxyindole; Quinone chemistry

1. Introduction

Upon wounding or microbial invasion, insects mount a battery of immune responses involving plasma factors, hemocytes, and fat body (Gillespie et al., 1997; Lavine and Strand, 2002; Kanost et al., 2004). The latter two are responsible for a majority of the defense molecules released to the circulation or located on the immune cell surface. These molecules include antimicrobial peptides that directly kill micro organisms and proteins/enzymes that recognize pathogens, mediate and modulate defense signals, or generate reactive chemicals for wound healing and pathogen killing. Although genetic and biochemical analyses allow us to dissect some of these processes, intricate molecular interplays often make it difficult to determine the relative

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efficacies of individual responses. For instance, due to high chemical reactivity and possible cytotoxicity of quinones, the proteolytic activation of prophenoloxidases (proPOs), which generates phenoloxidases (POs) for quinone production, has long been considered as an important mechanism for pathogen immobilization and extermination (Sugumaran, 2002; Cerenius and Söderhäll, 2004; Nappi and Christensen, 2005). Yet, since other molecules in the plasma can kill microbes and active PO has never been isolated for in vitro analysis, there is no direct experimental evidence indicating that PO-generated reactive compounds contribute to the destruction of microbial cells. In fact, Leclerc et al. (2006) recently demonstrated that the resistance to microbial infection was not impaired in the *Drosophila* PAE1 mutant, which lacked a proPO-activating enzyme. Does active PO play any role in the killing of microorganisms?

Insect POs, or tyrosinase-type POs, are similar to mammalian tyrosinases with two catalytic activities: the oxygenase activity hydroxylates monophenols to *o*-diphenols and the oxidase activity converts *o*-diphenols to quinones (Sugumaran, 2002; Nappi and Christensen, 2005). Due to their broad substrate specificity (Aso et al., 1984; Hall et al., 1995), POs may participate in multiple steps of melanin formation, wound healing, cuticle sclerotization, and microbe killing (Fig. 1) (Ashida and Brey, 1997; Sugumaran, 2002; Nappi and Christensen, 2005). In vitro and in vivo studies demonstrated that PO generates quinones for melanogenesis (Jiang et al., 1997; De Gregorio et al., 2002a) and leads to changes in the physical properties of hemolymph clot through protein crosslinking (Bidla et al., 2005). Cuticle tanning (i.e. pigmentation and sclerotization) was attributed to a laccase—rather than tyrosinase-type PO in *Tribolium castaneum* (Arkane et al., 2005). Therefore, we decided to test the controversial function of PO by directly measuring possible antimicrobial activity of the reactive compounds produced in vitro by this enzyme. We used purified *M. sexta* proPO-activating proteinase-3 (PAP-3) and serine proteinase homolog-1 and -2 (SPH-1 and SPH-2) to activate proPO—the SPHs lack active site Ser or catalytic activity but are required to accompany PAP and proPO in the cleavage activation reaction (Kwon et al., 2000; Yu et al., 2003; Wang and Jiang, 2004; Gupta et al., 2005). Active PO generated quinones from its natural substrates. After treating microbes with the reaction mixtures, we determined morphology, survival, and growth of the cells and established that the reactive intermediates yielded by PO had a broad-spectrum bactericidal activity. In addition to that, we identified the reaction products that kill the microbes.

2. Materials and methods

2.1. Chemicals, proteins, and microbial strains

Dopamine, *L*-dopa, and 1-phenyl-2-thiourea (PTU) were purchased from Sigma. *N*-acetyldopamine (NADA) and *N*- β -alanyldopamine (NBAD) were generously provided by Dr. Kanost in Department of Biochemistry at Kansas State University. DHI, or 5,6-dihydroxyindole, was supplied by Chemical Synthesis and Drug Supply Program at National Institute of Mental Health. *M. sexta* proPO was purified from hemolymph of the 5th instar larvae (Jiang et al., 1997). *M. sexta* PAP-3, SPH-1, and SPH-2 were isolated from plasma of the bar-stage wandering larvae as described previously (Jiang et al., 2003; Wang and Jiang, 2004). *E. coli* X11blue strain (Stratagene) was transformed with pGB5 (Bloemberg et al., 1997), a GFP-expressing plasmid kindly provided by Dr. O'Toole in the Department of Microbiology and Immunology at Dartmouth Medical University. Nonpathogenic strains of *B. cereus*, *B. subtilis*, *K. pneumoniae*, *M. luteus*, *P. aeruginosa*, *S. cerevisiae*, *S. typhimurium*, and *S. aureus* were obtained from Department of Microbiology at Oklahoma State University. *B. bassiana* and *C. albicans* were kindly provided by Drs. Stephen Marek and Guolong Zhang at Oklahoma State University.

2.2. Preparation of microbial cells

A single colony of each bacterial strain was inoculated to 3 ml Luria-Bertani (LB) medium containing appropriate antibiotics and grown at 37°C for 12 h at 225 rpm. *P. pastoris*, *C. albicans*, *S. cerevisiae*, and *B. bassiana* were cultured in 3 ml YEPD medium at 28°C for 16 h at 260 rpm. The overnight cultures were centrifuged at 2500g for 5 min. After careful removal of all the supernatants, cell pellets were separately resuspended in sterile phosphate-buffered saline (PBS: pH 7.4, 10mM sodium phosphate, 150mM NaCl) to a final A₆₀₀ of 1.0.

2.3. ProPO activation and substrate selection

Purified proPO (0.2 µg/µl, 2 µl), PAP-3 (30 ng/µl, 2 µl), and SPHs (30 ng/µl, 2 µl) were added to 14 µl, 20mM Tris-HCl, pH 7.5 in a microplate well. After incubation on ice for 1 h, the activation mixture was reacted with 150 µl, 50mM sodium phosphate (pH 6.5) containing 2mM L-dopa, dopamine, NADA, NBAD, or DHI. Quinone and melanochrome formation was measured every 15 s for 5 min at 350 and 470 nm, respectively, on a VersamaxTM microplate reader (Molecular Devices).

2.4. Effect of dopamine-derived compounds on the growth of microbial cells

Resuspended *E. coli*, *B. subtilis*, or *P. pastoris* cells (5.0 µl) were separately added to 20 µl proPO activation mixture (i.e., PO) and 150 µl dopamine solution. As controls, the cell suspensions were also treated with: (1) PO and 150 µl buffer (50mM sodium phosphate, pH 6.5), (2) buffer (20 µl) and dopamine solution, and (3) PTU-treated PO (~20 µl) and dopamine solution. After incubation at 25 °C for 1.5 h, 120 µl of the samples were inoculated to 4.0 ml medium (*E. coli* carrying pGB5: LB containing 100 µg/ml ampicillin and 100 µg/ml kanamycin; *B. subtilis*: LB; *P. pastoris*: YEPD) and grown at 37 °C with shaking. For the bacteria, A₆₀₀ readings were taken at 1–2 h intervals for 4–10 h. For the yeast, absorbance was measured once every 4h for 1 day.

2.5. Phase-contrast and fluorescence microscopy

After *E. coli* and *B. subtilis* cells had been treated with dopamine in the presence or absence of PO for 1 h, 10 µl of the reaction and control mixtures were added onto a glass slide. The bacterial cells were observed and photographed with phase-contrast or fluorescence optics under an Olympus BH-2 microscope.

2.6. Microbial colony counting

Following treatment by dopamine with or without PO for 16 h, *E. coli* and *B. subtilis* cells in the reaction and control were diluted with sterile PBS to 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, and 10⁻⁷. Diluted cell suspensions (20 µl each) were spread to marked areas (1 cm × 1 cm) on LB agar plates containing appropriate antibiotics. The dried plates were then inversely incubated at 37 °C for 12 h (*B. subtilis*) or 18h (*E. coli*). Additionally, the treated and control bacteria were sonicated in a water bath (Fisher, model FS60) for 2.5 min to disperse cell aggregates. After aggregate dissociation was confirmed by microscopy, serial dilution and colony growth were performed as described above. The same experiment was carried out to test the survival of the other bacteria: *B. cereus*, *M. luteus*, *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, and *S. typhimurium*. Dependent on their growth rates, the LB plates were incubated for 6–48 h so that colonies had a proper size for counting. Effect of DHI on the survival of bacteria (*E. coli*, *B. subtilis*) and fungi (*P. pastoris*, *S. cerevisiae*, *C. albicans*, and *B. bassiana*) were examined similarly after the microbes had been treated with 150 µl PBS containing DHI (2mM) alone, DHI and vitamin C (2mM each), or DHI (2mM) and proPO activation mixture (20 µl).

3. Results

3.1. Enzymatic activity and substrate specificity of *M. sexta* PO

Pre-incubation of purified proPO, PAP-3, and SPHs generated active PO, as previously demonstrated (Wang and Jiang, 2004). The oxidase then converted dopamine, NADA, NBAD, and *L*-dopa to their corresponding quinones at comparable rates (Fig. 2B). While the quinones formed at similar rates (as indicated by absorbance increases at 350 nm), the production of melanochrome from dopamine occurred 7–10 times as fast as that from NBAD, NADA, or *L*-dopa (Fig. 2A). The acetyl or β -alanyl group on the side chain withdraws electrons and greatly slows down the intra-molecular nucleophilic substitution (Aso et al., 1984).

3.2. Effect of PO-generated reactive intermediates on the growth of microbial cells

After PO and dopamine had been incubated with *E. coli* cells, we observed that the bacterial growth was completely blocked (Fig. 3A). This activity appeared to be concentration-dependent since the growth inhibition was incomplete when half of the PO activity was used. *E. coli* growth was not affected after the cells had been treated with the substrate or PO alone. PTU inhibition of PO abolished the growth inhibition. PTU alone did not affect the bacterial growth (Fig. 3A and B). These results indicated that the products resulted from the PO–dopamine reaction are responsible for the growth inhibition. A similar effect was observed after *B. subtilis* cells had been treated with PO and dopamine. *B. subtilis* grew faster than *E. coli* and both bacteria were unaffected by the presence dopamine itself. In contrast, dopamine alone reduced the growth of *P. pastoris* (Fig. 3C). Inclusion of active PO in the substrate solution did further reduce the yeast growth, but the small effect may not be statistically significant. Treating the bacterial and fungal cells with PO and another natural substrate (NADA, NBAD, or *L*-dopa) has little effect on their growth (Fig. 3D).

3.3. Microscopic detection of melanized bacterial aggregates

We examined the morphology of *E. coli* and *B. subtilis* cells treated with dopamine only or dopamine and PO (Fig. 4). The control cells were smooth, transparent, and mostly isolated. On the contrary, large aggregates of the bacteria formed after incubation with the PO–dopamine reaction mixture. Parts of the cells were covered with black pigments and became rough. While movement of the control fluorescent cells was captured by photography during the exposure, PO-treated *E. coli* cells did not display such mobility even if they were not aggregated—the cell mobility was lost after the PO–dopamine treatment.

3.4. Bactericidal activity of PO-generated reactive intermediates

In order to test if there was any change in cell viability after the treatment, we measured the colony-forming units in the *E. coli* suspension containing dopamine only or dopamine and PO. There was a significant reduction in colony counts from 2.3×10^6 to 3.2×10^5 cfu/ml (Fig. 5A). Since cell aggregation (rather than direct killing) could also cause the decrease in CFUs, we sonicated the suspensions at a low power to disperse the aggregates. Viability of the control cells reduced 35% to 1.5×10^6 cfu/ml after sonication. While large aggregates in the treated cells were well separated after sonication (data not shown), the colony counts did not increase. On the contrary, we detected a 73% decrease to 8.6×10^4 cfu/ml—PO–dopamine-treated *E. coli* cells seemed to be more susceptible to sonication. These data suggested that PO-generated reactive compounds were responsible for aggregation and death of *E. coli*.

The antimicrobial activity was not limited to *E. coli*: more striking results were obtained when *B. subtilis* cells were tested (Fig. 5B). Before and after sonication, the cell mortality was determined to be 93% and 99% higher in the samples treated with PO and dopamine. The decrease was dependent upon cell density and treatment time (data not shown).

To examine specificity of this bactericidal effect, we tested other bacterial species: three Gram-positive and three Gram-negative (Table 1). *B. cereus* was most susceptible while *S. aureus* was most resistant: after PO–dopamine treatment and sonication, 99.7% and 52% of the cells died. In comparison, 60–94% of the Gram-negative bacteria were killed after the treatment.

3.5. Chemical nature of the bactericidal compounds

Why did dopamine greatly affect the bacterial survival (Table 1) and growth (Fig. 3A and B) but NADA or NBAD did not (Fig. 3D)? After comparing the structural differences between dopamine and its acyl derivatives (Fig. 1), we hypothesized that dopamine quinone or its subsequent reaction products had the bactericidal activity. Since dopamine and the other plasma catecholamines were all converted to their corresponding quinones (Fig. 2B), it seemed less plausible that dopamine quinone was responsible for the killing. Therefore, we tested the antimicrobial activity of DHI, a compound formed by spontaneous cyclization of dopamine quinone—NADA and NBAD quinones form DHI derivatives at a much lower rate (Aso et al., 1984).

In the absence of PO, DHI spontaneously oxidizes to form indole-5,6-quinone and melanochrome that polymerizes to become DHI eumelanin (Fig. 1). These reactions occurred at a ~30% higher rate in the presence of PO (Fig. 6A), confirming that *M. sexta* PO had a broad substrate specificity by acting on multiple steps of the quinone production and melanin formation (Hall et al., 1995; Sugumaran, 2002; Nappi and Christensen, 2005). DHI and/or its products resulted from spontaneous oxidation killed *E. coli* and *B. subtilis* more efficiently than dopamine-derived compounds—more than 99.9% of the bacteria died (Fig. 6B and C).

We also examined the effect of decrease or increase in the oxidation of DHI. While vitamin C itself did not affect the bacterial colony counts, its inclusion in the DHI solution slowed down the spontaneous oxidation (data not shown) and led to a 9.0% decrease in the *E. coli* mortality (Fig. 6B). To our surprise, an increase in the rate of oxidation by adding PO also gave rise to lower mortality rates (90.2% for *E. coli* and 99.8% for *B. subtilis*). These results are consistent with the observation that *B. subtilis* cells are more susceptible to the treatment than *E. coli* are (Table 1), and further suggested that DHI was the compound that killed the bacteria. Interestingly, DHI is a catechol rather than a quinone, which has long been assumed to be cytotoxic.

3.6. Antifungal activity of DHI

To investigate if the minor inhibition of *P. pastoris* growth (Fig. 3C) was due to limited amount of DHI produced in the PO–dopamine reaction (and its strong cell wall), we directly treated the yeast cells with DHI and observed significant growth inhibition (Fig. 7A). Viability tests showed DHI alone had the highest fungicidal activity (92.9% mortality), and inclusion of vitamin C or PO lowered the effect (39.7% and 50.3% mortality) (Fig. 7B). We further examined the activity against other fungal species, including the entomopathogenic fungus *B. bassiana* (Fig. 7C). The mortality rates of *S. cerevisiae*, *C. albicans*, and *B. bassiana* after DHI treatment were 99.5%, 99.3%, and 90.19%, respectively.

4. Discussion

Prophenoloxidase activation, quinone production, and melanization often occur at the site of wounding or infection in insects and crustaceans (Gillespie et al., 1997; Cerenius and Söderhäll, 2004; Nappi and Christensen, 2005). While these series of reactions have been widely recognized as an integral part of the defense system, only circumstantial experimental evidence is available so far for its role in microbe killing. For example, melanotic encapsulation takes place in some mosquitoes that are refractory to parasites (Gorman and Paskewitz, 2001). One

electron transfer from dopa to oxygen could have happened to produce dopa semiquinone and superoxide anion during melanization (Nappi et al., 1995). The superoxide then led to the formation of hydroxyl radicals that kill the parasitic wasp *Leptopilina boulardi* in *Drosophila*. PO activity in the larval hemolymph positively correlated with the virucidal effect of *Heliothis virescens* (Shelby and Popham, 2006).

Nevertheless, the precise role of PO and its reactive products in the fight against microbial infection has never been experimentally established due to several reasons. Coexistence of other defense molecules and mechanisms makes it difficult to assess the contribution of melanization through in vivo experiments. Because PO often associates with other proteins, sticks to column matrices, and loses its catalytic activity (Sugumaran, 2002), this enzyme has never been purified from any arthropod species to near homogeneity for in vitro tests. PO, as an enzyme with broad substrate specificity (Aso et al., 1984; Hall et al., 1995), produces many products that could be chemically synthesized for functional analysis. However, high reactivity and rapid conversion of these compounds make this approach inapplicable. Consequently, the significance of proPO activation, quinone toxicity, and melanin production in arthropod immunity remains as a hypothesis lacking direct experimental support.

Using several purified proteins, we demonstrated for the first time that PO-generated reactive compounds immobilize, aggregate, and kill a variety of bacteria and fungi (Fig. 3–Fig. 7, Table 1). Proteolytic activation of proPO and PO-mediated production of reactive intermediates, therefore, are indeed an integral component of the insect immune system against microbial infection. Since proPO activation and PO activity have been observed in all the insect and crustacean species studied (Gillespie et al., 1997; Ashida and Brey, 1997; Cerenius and Söderhäll, 2004), we speculate that PO-catalyzed formation of DHI, quinones, and semiquinones is a universal defense mechanism to kill microbial pathogens. While semiquinones may associate with the generation and conversion of cytotoxic free radicals (Nappi and Christensen, 2005; Sugumaran, 2002), melanization is a common manifestation of these killing mechanisms.

To establish the function in vivo, we need to interpret these results based on physiological parameters of the plasma samples. In the larval, pupal, and adult hemolymph of *M. sexta*, NADA, or NBAD represents the primary catecholamine which, after oxidation, cyclizes 40 times more slowly than dopamine quinone (Aso et al., 1984; Hopkins et al., 1984). As major precursor of cuticle sclerotization, NADA or NBAD did not display any antimicrobial activity (Fig. 3D). Dopamine, the second most abundant catecholamine in the hemolymph, appears to be more important in the antimicrobial response. The maximal PO activity in the larval plasma is 5–10 times higher than that generated in vitro (Fig. 2). Conversion of *L*-dopa to dopamine by dopa decarboxylase (Nappi et al., 1992a) and DHI generation catalyzed by dopachrome conversion enzyme (DCE) (Johnson et al., 2001; Nappi et al., 1992b) may further enhance PO-mediated DHI production (Fig. 1). On the other hand, since the dopamine concentration in the hemolymph is only ~1/10 of that used in our functional tests (Hopkins et al., 1984), the role of dopamine in killing microorganisms is probably limited in vivo.

Obviously, we are unable to estimate the substrate concentrations and enzyme activities required for an effective, local antimicrobial response against pathogens from the environment, but there are several lines of evidence suggesting that DHI production and melanization play a critical role in insect defense responses. DHI was detected in the hemolymph of immune reactive larvae of *Drosophila* parasitized by *L. boulardi*, but not in the parasitized susceptible larvae or unparasitized larvae (Nappi et al., 1992b). *DCE1* mRNA level increased in adult females *Aedes aegypti* when melanotic encapsulation occurred after inoculation of microfilariae (Johnson et al., 2001). The PO and dopa decarboxylase activities were elevated

in *D. melanogaster* and *Armigeres subalbatus* during melanization (Nappi et al., 1991; Huang et al., 2005).

Although the cytotoxicity of DHI to host cells has not been measured, we suggest that a low concentration of dopamine largely limits the detrimental effect of excessive melanization. This may also explain why in vivo experiments failed to demonstrate the role of melanization in microbe killing (Leclerc et al., 2006). In the fly-survival tests, the number of bacteria used to infect adult *Drosophila* was overwhelmingly high and probably over-represented the challenge faced by the flies in their natural habitats. We further noticed, while there was no major decrease in the survival rate of *PAE1* mutant, significant reduction was found in the *Bc* mutant that lacks plasma PO (Leclerc et al., 2006). In comparison to the *spätzle* and *relish* mutants, this reduction was not as prominent. However, since over 100 immunity-related genes were affected in these Toll pathway mutants (De Gregorio et al., 2002b), it is perhaps inappropriate to use those as references to assess the contribution of melanization. During the preparation of this paper, we noticed that Tang et al. (2006) further evaluated the roles of *Drosophila* MP2 (i.e. PAE1) and MP1 in melanization. RNA interference coupled with survival tests indicated that melanization was crucial, but not essential, for resistance to bacterial and fungal infection in flies impaired in the Toll or imd pathway.

Insect innate immunity consists of both cellular and humoral responses that are intricately associated. Differences in property of Cathogens/parasites, route of infection, physiological state, and developmental stage may dramatically change the efficacies of individual responses. Our study implied a critical role of proPO activation and DHI production in antimicrobial responses. Further investigations are required to better understand the biochemical functions of serine proteinases, dopa decarboxylase, PO, and DCE in insect immunity.

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Abbreviations

proPO and PO, prophenoloxidase and phenoloxidase; PAP/PAE, proPO-activating proteinase/enzyme; PTU, 1-phenyl-2-thiourea; LB, Luria-Bertani; PBS, phosphate-buffered saline; GFP, green fluorescent protein; NADA, *N*-acetyldopamine; NBAD, *N*- β -alanyldopamine; DHI, 5, 6-dihydroxyindole; DDC, dopa decarboxylase; DCE, dopachrome conversion enzyme; MP, melanization proteinase.

Fig 1.

Mechanisms and physiological functions of PO-mediated reactions in insects and crustaceans. Tyrosinase-type POs, along with other enzymes, generates dopamine quinone which rapidly cyclizes to form DHI nonenzymatically. DHI, as well as its oxidation products, has a broad-spectrum antimicrobial activity (this work). These products spontaneously polymerize to form melanin frequently observed around wounds, pathogens, or parasites. Dopamine can also form stable derivatives through acyl transfer. Laccase-type POs then convert NADA and NBAD to oxidative intermediates for protein crosslinking during wound healing and cuticle sclerotization.

Fig 2.

Progressive curves for the PO-catalyzed conversion of phenolic compounds, monitored at 470 nm (A) and 350 nm (B). As described in the Materials and Methods, active PO generated by incubation of proPO with PAP-3 and SPHs was incubated with *L*-dopa (----), dopamine (● — ●), NADA (+ — +), or NBAD (□ — □).

Fig 3.

Effect of PO-generated reactive intermediates on the growth of *E. coli* (A), *B. subtilis* (B,D) or *P. pastoris* (C). Panels A–C: after the cell suspensions were treated with dopamine (▼ — ▼), PO (○ — ○), PTU only (△ — △), dopamine with PO (□ — □), half of PO (■ — ■), or PTU-treated PO (× — ×), fresh media were added to the cells. Absorbance readings were taken from the cultures at the different intervals and plotted against time. Panel D: *B. subtilis* cells treated with NADA (□ — □), NADA and PO (■ — ■), NBAD (△ — △), NBAD and PO (▲ — ▲), L-dopa (○ — ○), L-dopa, and PO (● — ●).

Fig 4.

Morphology, mobility, and association states of the bacterial cells examined under the phase-contrast (A, B, E, and F) and fluorescence (C, D) microscope. As indicated in the Materials and Methods, *E. coli* (A–D) or *B. subtilis* (E, F) were treated with dopamine only (A, C, E) or dopamine and PO (B, D, F) at room temperature for 16 h. The suspensions were added to a glass slide and photographed with different optics. The length of scale bar is equivalent to 10 μm .

Fig 5.

Effect of PO-generated reactive compounds on viability of *E. coli* (A) and *B. subtilis* (B). After the bacterial cells were treated with dopamine only or dopamine with PO at room temperature for 2 h, the suspensions were either directly diluted and grown on solid medium or sonicated before serial dilution and culturing. The colony counts (cfu/ml) were plotted in the bar graph as mean $\pm$ SD ($n = 4$).

Fig 6.

PO-catalyzed DHI oxidation (A) and the bactericidal activity of DHI (B, C). (A) Progressive curve. After DHI was added to PBS or active PO, absorbance was monitored at 350 and 470nm on a plate reader. DHI and PO at 350nm (○ — ○); DHI only at 350nm (● — ●), DHI and PO at 470nm (□ — □); DHI only at 470nm (▲ — ▲). (B) *E. coli* and (C) *B. subtilis*. After the bacterial cells were treated with PBS, vitamin C, DHI, DHI + vitamin C, or DHI + PO at room temperature for 1 h, the cell suspensions were sonicated, diluted, and grown on solid LB medium. The colony counts (cfu/ml) were plotted in the bar graph as mean±SD ($n = 4$).

Fig 7.

Effect of DHI on the survival and growth of fungi. (A) *P. pastoris* growth inhibition: vitamin C (●—●); PBS (□—□); DHI + vitamin C (▲—▲); DHI + PO (○—○); DHI only (◆—◆). (B) Killing of *P. pastoris*: after the yeast cells were treated with buffer, DHI, vitamin C, DHI + vitamin C, or DHI + PO at room temperature for 1 h, the cell suspensions were diluted and grown on solid YEPD medium prior to colony counting. (C) Antifungal activity against *S. cerevisiae*, *C. albicans*, and *B. bassiana*. Following buffer or DHI treatment, the fungal cells were sonicated, diluted, and grown on the solid medium. The colony counts (cfu/ml) were plotted in the bar graph as mean±SD ($n = 4$).

Table 1
Bactericidal activity of reactive compounds generated by *M. sexta*; phenoloxidase^a

Bacterial species	Colony counts		% of control
	Control	Treatment	
<i>Gram positive</i>			
<i>Bacillus cereus</i>	$5.2 \pm 2.3 \times 10^5$ ($n = 4$)	$1.4 \pm 2.4 \times 10^3$ ($n = 4$)	0.27
<i>Bacillus subtilis</i>	$9.8 \pm 8.2 \times 10^4$ ($n = 4$)	$1.2 \pm 2.4 \times 10^3$ ($n = 4$)	1.2
<i>Micrococcus luteus</i>	$5.8 \pm 1.6 \times 10^6$ ($n = 4$)	$3.0 \pm 3.6 \times 10^6$ ($n = 4$)	52
<i>Staphylococcus aureus</i>	$1.4 \pm 0.1 \times 10^6$ ($n = 4$)	$6.8 \pm 1.4 \times 10^5$ ($n = 4$)	48
<i>Gram negative</i>			
<i>Escherichia coli</i>	$1.5 \pm 0.6 \times 10^6$ ($n = 4$)	$8.6 \pm 3.0 \times 10^4$ ($n = 4$)	5.7
<i>Klebsiella pneumoniae</i>	$1.8 \pm 1.8 \times 10^8$ ($n = 4$)	$1.3 \pm 0.7 \times 10^7$ ($n = 4$)	7.2
<i>Pseudomonas aeruginosa</i>	$1.3 \pm 0.1 \times 10^7$ ($n = 4$)	$2.1 \pm 1.2 \times 10^6$ ($n = 4$)	16
<i>Salmonella typhimurium</i>	$2.4 \pm 0.9 \times 10^7$ ($n = 6$)	$9.6 \pm 0.9 \times 10^6$ ($n = 5$)	40

^a Overnight bacterial cultures were treated with PO and dopamine or the substrate alone (control) at room temperature for 18 h. After sonication and serial dilution, colony-forming units (cfu) per ml in the incubation mixtures were determined as described in the Experimental Procedures. The results are presented as mean $\pm$ CI ($\alpha = 0.05$, $n = 4-6$) and percentage of survival.