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Phenoloxidase and Melanization Innate Immune Activities in Green Darner Dragonfly Nymphs (*Anax junius*)

Mark Merchant[1*], Matthew R. McClure[2]

[1]Department of Chemistry, McNeese State University, Lake Charles, USA
[2]Department of Biology, Lamar State College Orange, Orange, USA
Email: *mmerchant@mcneese.edu, matt.mcclure@lsco.edu

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Abstract

Insects in the Order Odonata are highly subject to infection by gregarine parasites. However, despite the important ecological roles that insects play in every ecosystem in which they exist, little research has been devoted to the description of insect immunity. Insects rely heavily on the rapid actions of innate immune mechanisms to prevent infection. We characterized the melanization response in the hemolymph of green darner dragonfly (*Anax junius*) nymphs. Incubation of chymotrypsin-activated hemolymph with L-DOPA resulted in volume- and time-dependent production of dopaquinone via the phenoloxidase (PO) enzyme, with biphasic accumulation of product. The PO activity was temperature-dependent, with a stepwise increase from 20˚C - 35˚C and maximum activity measured at 35˚C - 40˚C. The formation of product was also inhibited in a concentration-dependent manner by diethylcarbonate, a specific inhibitor of PO activity, which indicated that the observed activity was due to the presence of PO enzyme. The rate of formation and quantity of melanin was dependent on exposure to different titers of bacteria. This is the first characterization of both PO activity and melanization response in green darner dragonflies.

Keywords

Aquatic Insects, Arthropod, Dragonfly, Hemolymph, Innate Immunity, Insect

1. Introduction

All animals require host defense mechanisms to avoid infection and colonization by potentially infectious microbes. These immune mechanisms have the ability

to distinguish self from non-self tissues, can activate effector molecules to target potential infectious agents [1], and can be divided into two broad categories: innate immunity and adaptive immunity. While the adaptive immune system generally exhibits high specificity, it often requires multiple exposures and can take several days to develop a full response. In contrast, innate immunity displays nonspecific activities but acts immediately upon exposure as a rapid, first defense against invasion of microbes. These two systems often interact to produce an effective defense against microbial insult. While invertebrates do not rely entirely on innate immune mechanisms for host defense, their adaptive mechanisms of immunity are generally believed to lack the complexity of those that are common in vertebrates [2]. However, recent studies have shown that insect immunity may be more complex than first described [3]. Arthropods utilize a range of cellular and humoral defense strategies to prevent microbial infection. These means of protection are extremely efficient and effective at isolating, encapsulating, and clearing large numbers of infectious microbes [4].

Arthropods utilize multiple methods of host defense including the production of antimicrobial peptides, phagocytosis of microbes, and melanization [5]. The melanization response is probably the most rapid and nonspecific immunological response. It involves the immediate deposition of melanin, an insoluble black polymer of dopaquinone, on the surface of microbes or parasites until the insult is completely encased in a shell of melanin. This pathway employs a proteolytic activation of the zymogen prophenoloxidase (proPO) by a serine protease cascade which requires the interaction of a host pattern recognition protein, such as peptidoglycan recognition protein or β-1,3-glucan recognition protein, with pathogen target proteins [6] [7]. The activated phenoloxidase (PO) enzyme then catalyzes the oxidation of phenols to quinones which rapidly polymerize, in a non-enzymatic process, to form melanin [8]. The most common substrate for the formation of melanin is tyrosine [9]. It is interesting that this immunological mechanism seems to be restricted to arthropods, as no known vertebrates or other invertebrates exhibit this means of defense [10]. Melanization effectively neutralizes immunological threats from parasites, bacteria, fungi, and viruses [11].

The expression of phenoloxidase enzyme activity has been positively correlated with the resistance against a variety of microbial pathogens [12]. In addition, host insects with high PO activities have been shown to have lower parasite loads [3] and increased survival of microbial infections [13]. Furthermore, insect strains that are deficient in the melanization response typically have decreased resistance to infection [14]. Therefore, PO activity and subsequent melanization are thought to be an integral part of insect immune defense, and the melanization response has been detected and compared across a variety of damselfly and dragonfly species [15].

Insects play important roles in a broad spectrum of ecosystems and habitats. They are important parts of the food web in virtually all ecosystems in which they exist [16]. Members of the Order Odonata (dragonflies and damselflies) can

often act as indicator species of the health and integrity of aquatic ecosystems, and sometimes also the adjacent riparian or littoral areas [17] [18]. They are also predators of insect disease vectors and agricultural pests [17]. It is important to understand resistance and susceptibility to infection and mechanisms of immunity in this important group of invertebrates. The focus of this study was to provide a detailed characterization of PO activity and melanization in hemolymph of the green darner dragonfly (*Anax junius*) nymph.

2. Materials and Methods

Chemicals and biochemicals—sodium cacodylate, $CaCl_2$, L-DOPA, and a-chymotrypsin were purchased from Millipore-Sigma (St. Louis, MO).

Collection of dragonfly nymphs—nymphs were collected in heavy emergent vegetation in shallow marsh habitats using insect collection nets. Eighteen nymphs were collected from fish research ponds on the campus of the University of Illinois in Champaign, IL, and three late instar nymphs were collected from the wetland demo garden at Shangri La Botanical Gardens and Nature Center in Orange, TX. The nymphs were placed individually in cups full of water from the environment in which they were captured. The species of nymphs were positively identified using a specific manual for the identification of dragonfly larvae [19].

Collection of hemolymph—nymphs were maintained in natural marsh water (22˚C - 23˚C) aerated with a pump and air stone until hemolymph was to be collected (generally less than 12 hrs.). A 26 ga needle attached to a 1.0 mL syringe was inserted between the dorsal abdominal tergites of segments 7 and 8 to right side of the midline. Hemolymph was collected and transferred to a 500 mL microcentrifuge tube in an ice bath. The hemolymph was flash frozen in a dry ice/ethanol bath and frozen at −80˚C until ready for phenoloxidase or melanization assays.

Phenoloxidase assays—for the volume-dependent activity, various volumes of hemolymph (0 - 20 mL) were balanced with assay buffer (10 mM sodium cacodylate, 10 mM $CaCl_2$, pH 8.4) to a total volume of 50 mL. The diluted hemolymph was treated with 10 mL of α-chymotrypsin (1 mg/mL) for 20 min. at ambient temperature (~25˚C). This mixture was incubated with 40 mL of saturated L-DOPA in assay buffer in a 96-well plate, the reaction was allowed to proceed for 30 min., and the optical density was measured every 2 min. At 490 nm using a BioRad Benchmark™ plate reader.

To determine the effects of temperature on PO activity of hemolymph, 100 mL of hemolymph were mixed with 500 mL of assay buffer (10 mM sodium cacodylate, 10 mM $CaCl_2$, pH 8.4) were treated with 100 mL of α-chymotrypsin (1 mg/mL) for 20 min at ambient temperature (~25˚C). The treated hemolymph (75 mL) was placed in wells of a microtiter plate and equilibrated at either 5˚C, 10˚C, 15˚C, 20˚C, 25˚C, 30˚C, 35˚C or 40˚C for 10 min. The reaction was initiated with 50 mL of saturated L-DOPA in assay buffer and the absorbance was measured at 490 nm after 30 min. using a BioRad Benchmark™ plate reader.

Melanization assays—to measure melanization of hemolymph in the presence of different concentration of bacteria, 30 mL of hemolymph diluted with 75 mL of assay buffer (10 mM sodium cacodylate, 10 mM $CaCl_2$, pH 8.4) were added to 50 mL of different solutions of *E. coli* (10^7, 10^6, 10^5, 10^4, or 10^3 CFU/mL suspended in sterile saline) in different wells of a 96-well plate. The optical density at 600 nm was measured immediately to provide a baseline absorbance, and then measured every 60 seconds to monitor melanization using a BioRad Benchmark Plus™ plate reader.

Statistics and controls—each data point in each assay represents the mean ± standard deviation of four independent determinations. The concentration of dopaquinone was calculated using a molar extinction coefficient of 3600 $M^{-1}\cdot cm^{-1}$. The concentration of melanin was calculated using a molar extinction coefficient of 11 cm^{-1} $(mg/mL)^{-1}$ [20].

3. Results

The formation of dopaquinone (DOPA) by the phenoloxidase enzyme in the hemolymph of the green darner dragonfly nymph was volume- and time-dependent (Figure 1(a)). The formation of DOPA was biphasic, with an initial rapid formation of product for approximately 7 minutes, followed by a slower linear

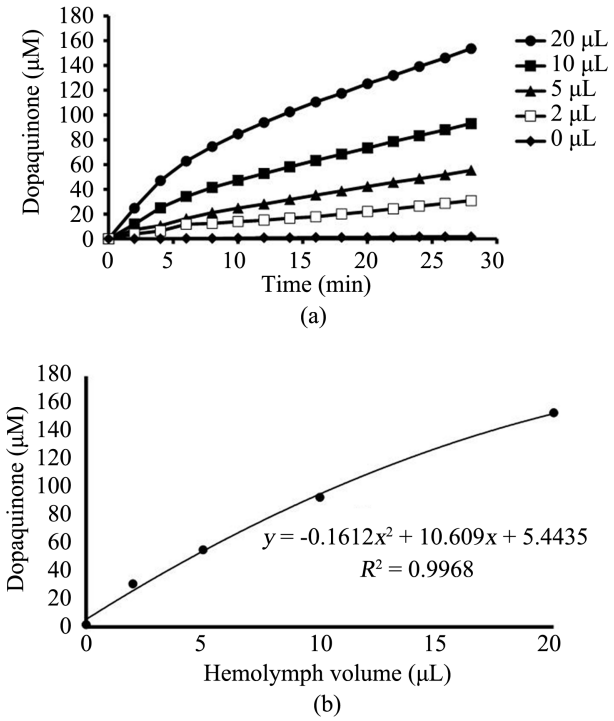

Figure 1. Volume-dependent formation of DOPA by hemolymph from green darner dragonfly nymphs. A. PO activity increased in a biphasic manner with increased in hemolymph volume. Results represent the means of four independent determinations. B. Kinetic analysis of the concentration-dependent production of DOPA by hemolymph from green darner dragonfly nymphs. The parabolic curve with the polynomial fit shows the nonlinearity of the increase in PO activity with volume.

accumulation of product. Inclusion of 2, 5, 10 or 20 mL of hemolymph resulted in the formation of 30.8, 55.3, 93.1, or 153.6 mM product after 28 min of incubation, respectively. The inclusion of assay buffer with no hemolymph resulted in no significant absorbance at 490 nm (<1.3 mM) at any time point, which indicated that there was little or no spectrophotometric interference in the assay. When the formation of product was plotted against the volume of hemolymph used, a parabolic curve of the 2nd order polynomial fit resulted, with a R^2 value of 0.9968 and a y-intercept of 5.4435 mM (Figure 1(b)).

Incubation of *α*-chymotrypsin-treated hemolymph with L-DOPA at different temperatures resulted in temperature-dependent formation of product. The enzymatic activities were relatively low (117.0 - 131.6 mM product accumulated) below 25˚C, with a stepwise increase from 20˚C - 30˚C. Peak activities of 177.1 ± 17.1 and 177.1 ± 6.2 were measured at 30˚C and 35˚C, respectively (Figure 2).

The PO-mediated accumulation of L-DOPA product in dragonfly hemolymph was measured at 352.6 ± 31.6 mM (Figure 3). The addition of diethylthiocarbamate (DETC), a specific inhibitor of PO activity, reduced activity in a concentration-dependent fashion. The activity was inhibited 24.4% ± 4.8%, 21.2% ± 4.6%, and 51.2% ± 7.5% by 2, 8, and 40 mM (DETC).

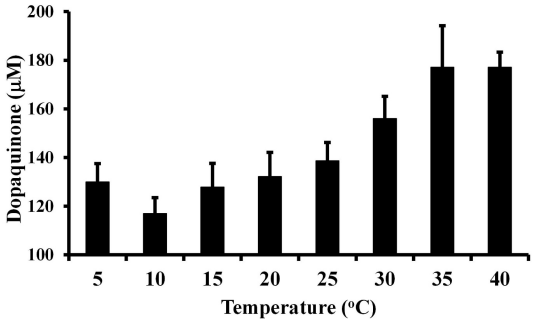

Figure 2. Thermal profile of the concentration dependent production of DOPA by hemolymph from the green darner dragonfly. Results represent the means ± standard deviations of four independent determinations.

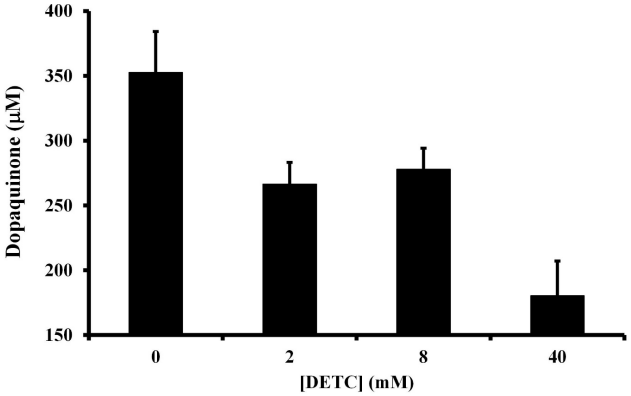

Figure 3. Concentration-dependent inhibition of PO activity by diethylthiocarbamate in the hemolymph of the green darner dragonfly. Results represent the means ± standard deviations of four independent determinations.

Incubation of different titers of *E. coli* bacteria with the diluted hemolymph of green darner dragonfly nymphs resulted in different rates of melanization (Figure 4). The production of melanin was asymptotic, with linear production of product 6 - 7 minutes followed by a slowing rate of melanization. Incubation of 10^3, 10^4, 10^5, 10^6, or 10^7 bacteria/mL produced peak concentrations of 8.6, 11.8, 14.4, 18.0, or 19.9 mg/mL melanin, respectively.

4. Discussion

Adaptive immunity is a more advanced system of host defense and is thought to have developed in early fishes (agnathans and gnathostomes, [21]), while innate immunity is more basal and is known to have been present in cephalochordates some 600 - 650 mya.

Although invertebrates have immunoglobulin-like molecules that may represent precursors to modern host protection, they serve functions other than immunological defense [22] [23] [24] and lack the ability to rearrange to form a myriad of diverse antigen binding sites as in vertebrates [25]. However, several studies have identified diverse immunoglobulin-like proteins in insects [26] [27]. In lieu of an advanced adaptive immunity, these invertebrates exhibit well-developed innate immune systems [28] [29] [30]. In the absence of complex acquired immunity, the immune system evolved innate systems of pattern recognition for the detection of non-self substances as organisms developed systematic ways of recognizing recurring molecular microbial patterns distinctly different from host antigens [31]. One such mechanism of innate host defense involves the monophenoloxidase enzyme, which catalyzes the oxidation of phenol to dopaquinone, which then spontaneously polymerizes in a process called melanization to deposit insoluble melanin and encapsulate microbes and parasites. This mechanism of immunological defense, which is thought to have evolved some 600 mya [1] and before the split between protostomes and deuterostomes [32], is present in early invertebrates such as arthropods, mollusks, annelid, echinoderms, tunicates, and cephalochordates [33], is a simplistic but effective way of

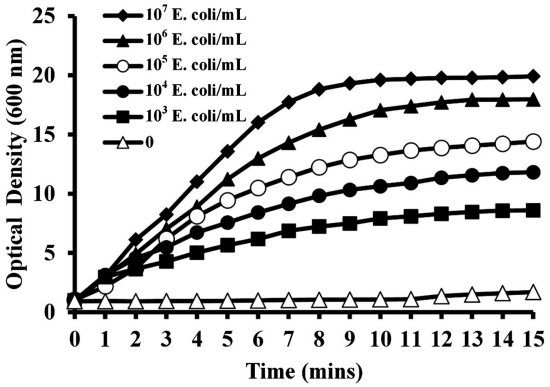

Figure 4. Kinetic analysis of melanization in hemolymph of the green darner dragonfly exposed to different concentrations of *E. coli* bacteria. Results represent the means of four independent determinations.

isolating internal threats, thus insulating and protecting the host organism via physical separation by a sheath of melanin [10]. The measurement of melanization can provide an indication of the degree of immune competence [34] [35] [36] and potentially the overall fitness [37] of an organism, as immunological threat can negatively impact other important physiological functions such as digestion, growth, reproduction, etc. The activation of PO activity of hemolymph from the nymph of the green darner dragonfly is rapid and concentration-dependent (**Figure 1(a)**).

Ectothermic animals have limited biochemical means of thermoregulation, and thus generally utilize behavioral mechanisms to exploit their external thermal environments. However, several studies have shown both behavioral and physiological thermoregulation strategies in several species of dragonflies [38]. These animals can increase internal temperature by increasing wingbeat frequency to produce metabolic heat, derived from the flight muscles, which can increase thoracic temperatures that are substantially warmer than ambient air temperatures [39]. In addition, dragonflies can rest in different thermal environments (sun or shade) and use a variety of body and wing postures and orientations relative to the direction of solar radiant energy [39]. Like all ectothermic organisms, their physiology and biochemistry are temperature-dependent. The PO activity in dragonfly hemolymph was found to be maximal at 35˚C - 40˚C (**Figure 2**). This temperature range almost exactly matches the thermal body temperature preferences of several species of dragonflies reported by [38]. Rapid responses of the innate immune system are important to prevent colonization by potentially-infectious microbes, and thus maintaining body temperatures in this range may be important for immunological fitness of these organisms. It is also likely that, in addition to immune function, which other physiological systems (digestive, circulatory, respiratory, etc.) are also maximal in this temperature range. At lower temperatures (5˚C - 20˚C), microbes do not grow as rapidly, and thus at temperatures below 25˚C infection poses a smaller threat and thus the lower activity of PO at these temperatures may be less immunologically important. However, dragonflies can rapidly increase thoracic temperatures by wing-whirring, which is a behavior during which dragonflies employ rapid contractions of wing muscles to produce metabolic heat [40] [41].

The activation of PO activity, and ultimately melanization, relies on the initial detection of conserved patterns of molecular patterns expressed by microbes. These detection mechanisms include Toll-like receptors and their downstream proteolytic cascades [42]. Insects express a full complement of functional Toll-like receptors [43]. Although the Toll receptors have ancient evolutionary history as they appear in different metazoan groups 700 mya [44], insects underwent an expansion of this family of immune receptors some 250 - 300 million years later [45] [46]. It is interesting that activation of PO activity can be achieved in hemolymph. It is known that activation of PO can be accomplished by soluble PPRs in insects [47]. The kinetics of PO activity (**Figure 1(a)**) was almost iden-

tical to that of melanization (**Figure 4**) in the hemolymph of the green darner dragonfly nymphs. The melanization response shows a similar kinetic curve (**Figure 4**). The response of soluble PPRs to activate an immune response may be important for organisms that utilize an open circulatory system in which organ systems are bathed in hemolymph.

Some Odonate species are in sharp decline on large scales due to the use of insecticides [48], infection and disease [49], changes in agricultural practices [50], and habitat loss [51]. In addition, localized extirpations are also a problem [52]. Dragonflies seem to be particularly susceptible to parasitism by gregarine parasites [53] [54] [55]. In addition, some dragonfly species have been implicated as reservoir hosts of chytrid fungus (*Batrachochytrium dendrobatidis*), which has had far-reaching detrimental population effects on amphibian populations worldwide [56]. Because these animals can be used as sentinel species to monitor the health of an ecosystem, it is important to understand their mechanisms of immunity. This is the first characterization of both PO activity and melanization in a species of dragonfly. These baseline data are important and can be used for comparison to those collected from animals in disturbed habitats, diseased populations, or comparison to other dragonfly species. The data presented in this study should provide a good reference for future studies that focus on the investigation of the effects of anthropogenic disturbances on the immune systems of green darner dragonflies.

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Conflicts of Interest

The authors declare that there are no conflicts of interest to disclose.

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Abbreviations

Colony Forming Units (CFU), Diethylthiocarbamate (DETC), L-3,4-dihydroxyphenylalanine, (L-DOPA), million years ago (mya), phenoloxidase (PO), prophenoloxidase (proPO)