Prophenol Oxidase Activation as an Insect Immune Response in Drosophila melanogaster

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Summary
The effects of phenol oxidase isoforms \( A_1 \) and \( A_3 \) on insect immunity was investigated in Drosophila melanogaster using two activity-null mutants, \( \text{Max}^{\text{SIMS}} \) and \( \text{Dox}^{-3\text{KDSS}} \), respectively. An insemination-dependent reaction involving innate immunity in the female uterus is among the self-defense reactions that inhibits interspecific hybridization between two related species, \( D. \text{nasuta} \) and \( D. \text{pallidifrons} \). The sequencing of prophenol oxidase provided new clues to identity of key components in innate immunity. Here I summarize how findings from genomic analysis contributed to information about the role of prophenol activation in innate immunity in Drosophila melanogaster.

Keywords: Insect Immunity, Prophenol Oxidase, Drosophila melanogaster

Introduction
All insects defend themselves against bacteria and parasites using cellular and humoral systems that are rapidly activated in the infected [5]. Among the induced effector molecules are antibacterial proteins and peptides [6]. Drosophila melanogaster is an excellent organism for using as a molecular and genetic approach to the study of insect immunity; and some these genes that are activated during the immune response have been cloned from this species. Major reactions, including activation of prophenol oxidase and clotting of the hemolymph, are immediately triggered by wounds or foreign objects in insects and other arthropods [9, 10]. Phenol oxidase (tyrosinase in mammal) catalyzes the key steps in the formation of the black pigment melanin, and prophenol oxidase activation results in a dark cuticle layer forming around wounds and encapsulation of parasites or, in extreme cases, a general darkening of the hemolymph in the Silkworm Bombyx mori [5], the Crustacean Pacifastacus leniusculus [9], and Drosophila melanogaster [1].

In Drosophila melanogaster, phenol oxidase comprises two prophenol oxidases, \( A_1 \) (tyrosinase, monophenol monooxygenase, EC 1.1.14.18.1) and \( A_3 \) (catechol oxidase, diphenol oxidase, EC 1.10.3.1) [8]. As a last step in phenol oxidase activation, a precursor enzyme is converted into an active form via a serine protease cascade in vivo [5] or via higher-order conformational and structural changes in vitro [4]. The effects of activity-null phenol oxidase mutants have been studied widely with regard to survival rates; two of these Drosophila melanogaster mutants, \( \text{Max}^{\text{SIMS}} \) (an \( A_1 \) mutant) and \( \text{Dox}^{-3\text{KDSS}} \) (an \( A_3 \) mutant), were isolated from natural populations in Gomel, Belorussia and Krasnodar, Russia, respectively. Other activity-null phenol oxidase mutants were isolated in our laboratory. In this article, I focus on the role of the prophenol oxidase system in insect immunity.

Materials and Methods

Flies and Chemicals
Drosophila melanogaster were reared on a standard cornmeal-yeast medium at 25 °C. The wild-type laboratory strain Oregon-R strain served as the control in each experiment. \( \text{L-tyrosine} \) (\( \text{L-}(p\)-hydroxyphenyl)alanine), \( \text{L-dopa} \) (\( \text{L-3,4-dihydroxyphenylalanine} \)), dopamine (3, 4-dihydroxyphenylethylamine), and bromanophenol blue die (6 x) were each purchased from Nakarai Tesque Inc. (Kyoto, Japan). Serine protease inhibitor Pefabloc (4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride) SC (AEBSF) was from Roche Diagnostics GmbH (Mannheim, Germany). The protein assay kit was from Bio-Rad Laboratory (Hercules, CA, U. S. A.), and the Fast Flow Liquid Chromatogram (FPLC) system from Pharmacia LKB Biotechnology (Uppsala, Sweden) was used to tentatively identify the prophenol oxidase protein.

Preparation of Prophenol Oxidase
The following procedures were performed at 0-4 °C unless otherwise specified. Centrifugation was performed at 16,000 rpm (30,000 g) for minutes in a Sakuma Model M-150 (Tokyo, Japan) centrifuge. Prophenol oxidase was collected in the supernatant after centrifugation and used as the starting material. The larvae were homogenized with a glass bar in sample buffer that contained 25 mM Tris-HCl, pH 7.2 at room temperature, 5 mL, 2-mercaptethanol, 20 mM homogenized with a glass bar in sample buffer that contained 25 mM Tris-HCl, pH 7.2 at room temperature, 5 mL, 2-mercaptethanol, 10 mL glycine, 2 g sodium dodecyl sulfate, bromophenol blue powder, and distilled water up to 50 mL. After a 2-minute centrifugation at

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16,000 rpm (30,000 g), the supernatant fraction, which included the prophenol oxidase A₁ and A₃ isoforms, was collected for characterization.

Native PAGE

For electrophoresis, polyacrylamide gel electrophoresis (native PAGE) with 7.5% gels was performed; activation of prophenol oxidase and determination of the phenol oxidase activity within the gel were performed as described [1]. For each sample, extract taken from a single pre-pupa was applied to a gel for electrophoresis.

Determination of Primary Sequencing and Three-Dimensional Structure

Edman degradation of purified A₁ and A₃ yielded no phenyl thiohydantain derivative of any amino acid showing a blocked amino terminus. Therefore, A₁ and A₃ were proteolytically digested. The resulting peptides were separated on a reversed–phase C18 HPLC column. The amino acid sequences were determined by the gas phase Protein Sequence System Procise model 492 (Perkin Elmer, Applied Biosystems Division, Foster City, CA, U. S. A). Given primary sequences were aligned and investigated.

The three-dimensional structure of prophenol oxidase protein was estimated using free WebLab Viewer software (Kitazato University, Tokyo, Japan) and molecular parameters were estimated using GENETYCS version 9 software (Software Development Co. Ltd., Tokyo, Japan).

Results and Discussions

Zymogram and Cross Experiments

In this article, the term ‘activation’ is used [1]. A typical zymogram (ca. 0.5 μg protein/lane) and phenol oxidase activated with 50% 2-propanol are shown in Figure 1. In pre-pupal extract from Oregon-R strain, a single band of phenol oxidase that corresponded to active A₁ was evident in the gel incubated with 20 mM L-tyrosine (lanes 1-3); two bands that corresponded to active A₁ and active A₃ were resolved when the gel was incubated with 20 mM L-dopa (lanes 4-6). In lanes with Mox⁴⁴⁹⁵ extract, no band or a faintly-stained band representing active A₁ was evident in the gel incubated with either 20 mM L-tyrosine (lane 2) or 20 mM L-dopa (lane 5). Similarly, in lanes with Dox-3KD₉₅ extract, no band that corresponded with active A₁ was evident in any gel (lanes 3 and 6). The zymogram showed that the visible marker genes had no effect on the expression of Mox⁴⁴⁹⁵ and Dox-3KD₉₅, therefore, these marker genes were expedient for calculating the gene frequency after mating experiments. Results of reciprocal mating experiment between activity-null mutants are shown in the following section.

Zymogram of Drosophila melanogaster prophenol oxidase A₁ and A₃ in a 7.5% polyacrylamide gel. Protein on the gel was activated with 50% 2-propanol, then treated with 20 mM L-tyramine (as a monophenol substrate, lanes 1-3) or 20 mM dopamine (as a diphenol substrate, lanes 4-6). Lanes 1 and 4: Oregon-R, lanes 2 and 5: Mox⁴⁴⁹⁵, lanes 3 and 6: Dox-3KD₉₅.

In the zymogram, black-colored bands showed final product melanized-melanin (lanes 1, 3-6) after homogenization (probably including room bacteria). No melanized sites (lanes 2, 5-6) were shown (probably including room bacteria). So homozygote with Mox⁴⁴⁹⁵ and Dox-3KD₉₅ was lethal [2] that plays as wound healing, sclerotization of cuticle resulting in a failure pupal formation as an insect immune response against antibody formation in mammals. Direct methods with different microbes were used to study Drosophila immune responses [7] and relied on genetically encoded factors recognized microbial features.

The protein content of A₁ in Mox⁴⁴⁹⁵ and A₃ in Dox-3KD₉₅ preparations was similar to that in the Oregon-R strain preparation after immunoblotting (data not shown) therefore differences between Oregon-R strain and each activity-null mutant with regard to phenol oxidase activity in the gel were not due to differences in the amount of enzyme protein between Oregon-R strain and each activity mutant.

Melanization is a common feature in both the cellular and humoral immune responses of insects; therefore, the substrates involved in melanin synthesis may be important in the non-self recognition processes in flies. Preliminary studies have shown a correlation between the defense reaction and phenol oxidase activity, epicuticular abrasion, or injection of five microorganisms, like bacteria. Here, I did not expose the animals to chemical irritants or mechanical abrasion; I only performed reciprocal mating to assess the importance of phenol oxidase. Results suggested that phenol
Phenol oxidase was indispensable for cuticle sclerotization and for self-defense against foreign matter in *Drosophila*.

To calculate the segregation values, about 4,400 individuals were scored for larva-to-adult viability. No adult individuals emerged in homozygous for A<sub>1</sub> in *Mox<sup>GM95</sup>* and A<sub>3</sub> in *Dox-3<sup>KD95</sup>*. Phenol oxidase is a bi-functional copper-retaining enzyme that exhibits monophenol oxidase and diphenol oxidase activities. Melanization is a common feature in both cellular and humoral immune responses of insects; therefore, the substrates involved in melanin synthesis may be important to the non-self-recognition processes in these insects.

The effect of activity-null mutation in genes encoding phenol oxidase on survival rate was investigated with *Mox<sup>GM95</sup>* and *Dox-3<sup>KD95</sup>*. *D. melanogaster* mutants; these structural genes encode prophenol oxidase A<sub>1</sub> and A<sub>3</sub>, respectively, and affect the phenol oxidase activity in them. Visible markers located on the second chromosome were linked by meiotic recombination to the mutations via mating experiments, and crosses were performed to generate double homozygous animals that carried mutation. No double mutant individuals emerged, suggesting that phenol oxidase and tyrosine-3-hydroxylase act as indispensable proteins to maintain life in *Drosophila*. Primary structure of prophenol oxidase was estimated by alignment (Figure 2).

Both enzyme-active sites (A and B, Figure 2) are highly conserved between the paralogs; the upstream A site is more conserved than the downstream B site, and there are six His residues around each site in each paralog [4]. Enzymes that use molecular oxygen to oxidize phenols have been prepared from various sources. These enzymes bind copper at the active center within a higher-order structure in *D. melanogaster*.

When the amino acid sequence of A<sub>1</sub> or A<sub>3</sub> is compared to those of other copper-containing proteins, such as vertebrate and microbial tyrosinase or arthropod hemocianins, it is clear that *D. melanogaster* prophenol oxidase contains two copper-binding sites, which correspond to two copper atoms per prophenol oxidase molecule. The two copper-binding sites, Cu (A) and Cu (B), have higher sequence similarity to hemocianin than to tyrosinase. This finding clearly indicates that the name phenol oxidase should be used for this *Drosophila* enzyme.

The viability of the double mutants (*Dox-3* and *Mox* double homozygotes) was greatly reduced relative to control populations: this finding indicated that phenol oxidase and tyrosine-3-hydroxylase act as essential proteins that are required for survival.

Alignment of deduced amino acid residues of *Dox-3* (A<sub>3</sub> protein) and *Mox* (A<sub>1</sub> protein) in *Drosophila melanogaster*. Arrow indicates the cleavage sites for activation of prophenol oxidase to active phenol oxidase. Arrow indicates the homology of amino acid

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**Figure 2.** Alignment of amino acid sequences encoded by *Dox-3* and *Mox*
residues. Shadows indicate two putative active sites, A site and B site.

These results suggested the three-dimensional structure (folding phase, diagram) of prophenol oxidase protein was derived from the primary structure and is presented as a ribbon-model (Figure 3).

Figure 3. Ribbon model diagram of prophenol oxidase A$_3$ (above) and A$_1$ (below) protein. Helical regions are shown α-helix ribbons.

Locations of N- and C-termini suggested that the resulting effects on catalytic function are equivalent to the construct of the enzymatic active center. The numbers of α-helices and β-sheets were estimated to be 14 and 8, respectively, in A$_3$; the numbers for A$_1$ isoform were unclear. The significance of the presence of a copper binding domain at the active center was that the center of the three-dimensional structure may be important for the catalytic phenol oxidase activity because one of the His ligands was involved in forming the substrate-binding domain. Each subunit may be viewed as having a rigid α-helix facing the active center; in contrast, the β-sheet regions were distributed around the C-terminal. The active center domains bound not at peripheral regions but at the center and were surrounded by the α-helix of the prophenol oxidase protein. The three-dimensional structure at the active center showed that each subunit contained six His residues that bound the two oxygen-binding Cu (II) atoms, which were separated by approximately 2.9 Å.

It is clear that during the phase leading to reproductive isolation between two related species, Drosophila nasuta and D. pallidifrons, D. nasuta females did not copulate with D. pallidifrons males. Nevertheless, D. pallidifrons females copulate with D. nasuta males at high frequency (approximately 70%); however, very few hybrid offspring result from these copulations; on average, eight individuals were produced for every 50 copulating females [3]. One suggested cause of the scarcity of hybrids was the insemination reaction and the consequent reaction-mass that forms when D. pallidifrons females mate with D. nasuta males. Oviposition may have been prevented by a reaction mass formation in the uterus, and this reaction and the innate immune reaction may have prevented hybrid formation between two species.

Conclusions

It is clear that recent research on the structure and function of immune factors has provided some clues as to the nature of non-self-recognition and cellular communication in insects, but it remains obvious that much work is needed before the complexity of these processes are fully understood and appreciated. Our recent research has been focused on iron metabolism and transport in Drosophila flies because, among other things, I have found that iron-transporting proteins seem to also be involved in immune defenses. Thus, I predict that Drosophila or any insects may serve as a good model for understanding how copper-containing and iron-containing proteins cooperate in the transport and delivery of oxygen to different tissues and cells without any production of oxygen radicals that are deleterious to all animals including humans.

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