Available online at www.sciencedirect.com
ScienceDirect
Developmental and Comparative Immunology 31 (2007) 879–888
Developmental & Comparative Immunology
www.elsevier.com/locate/devcompimm

Preliminary characterization of hemolymph coagulation in *Anopheles gambiae* larvae

Bogos Agianian[a,b], Christine Lesch[c], Olga Loseva[c,d], Mitchell S. Dushay[e,f,*]

[a]*European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany*
[b]*Department of Molecular Biology and Genetics, Democritus University of Thrace, Dimitras 19, 68100 Alexandroupolis, Greece*
[c]*Department of Molecular Biology and Functional Genomics, Stockholm University, 106 91 Stockholm, Sweden*
[d]*Department of Genetics, Microbiology, and Toxicology, Stockholm University, 106 91 Stockholm, Sweden*
[e]*Department of Life Sciences, Södertörns högskola, 141 89 Huddinge, Sweden*
[f]*Department of Comparative Physiology, Uppsala University, Norbyvägen 18A, 752 36 Uppsala, Sweden*

Received 30 November 2006; received in revised form 8 December 2006; accepted 18 December 2006
Available online 25 January 2007

Abstract

Hemolymph coagulation is a first response to injury, impeding infection, and ending bleeding. Little is known about its molecular basis in insects, but clotting factors have been identified in the fruit fly *Drosophila melanogaster*. Here, we have begun to study coagulation in the aquatic larvae of the malaria vector mosquito *Anopheles gambiae* using methods developed for *Drosophila*. A delicate clot was seen by light microscopy, and pullout and proteomic analysis identified phenoloxidase and apolipophorin-I as major candidate clotting factors. Electron microscopic analysis confirmed clot formation and revealed it contains fine molecular sheets, most likely a result of lipophorin assembly. Phenoloxidase appears to be more critical in clot formation in *Anopheles* than in *Drosophila*. The *Anopheles* larval clot thus differs in formation, structure, and composition from the clot in *Drosophila*, confirming the need to study coagulation in different insect species to learn more about its evolution and adaptation to different lifestyles.

Keywords: *Anopheles gambiae*; *Drosophila*; Coagulation; Lipophorin; Phenoloxidase; Insect immunity

1. Introduction

Coagulation is one of the first responses to injury in insects as in other animals, and the clot prevents infection as well as stops bleeding and contributes to wound healing (reviewed in [1–3]). While much is known about coagulation in *Limulus* [4], and studies have been done in other arthropods such as shrimp [5] and crayfish [6], the molecular mechanisms of coagulation in insects are not well understood. Studies have begun in *Drosophila melanogaster* larvae, where the clot has been described [7], and methods have been developed to isolate and identify candidate clotting factors [8,9]. Remarkably, few of these clotting factors have homologs in other insects [8]. This suggests that insect coagulation may have evolved through co-option of genes serving other functions [3,10]. The lack of conservation makes it important to study coagulation in different insects,

*Corresponding author. Department of Comparative Physiology, Uppsala University, Norbyvägen 18A, 752 36 Uppsala, Sweden. Tel.: +46 018 471 2805; fax: +46 018 471 6425.
E-mail address: mitch.dushay@ebc.uu.se (M.S. Dushay).

0145-305X/$ - see front matter © 2007 Published by Elsevier Ltd.
doi:10.1016/j.dci.2006.12.006

to learn how the clot is formed in different species, and by comparison to learn how evolution acted on this important defense process.

The mosquito *Anopheles gambiae* is the subject of intense interest because of its role as malaria vector; its humoral immune response has been studied [11–13], and genome published [14]. Although it is a dipteran insect like *Drosophila*, the two species are separated by 250 million years of evolution, and mosquito larvae are aquatic, so both time and different environmental constraints have acted on coagulation in these species. Coagulation in *Anopheles* is also of interest because some of its mechanisms may be shared with other immune reactions, including those against malaria parasites, and for the possibility that coagulation could provide a new target for pest control strategies.

We have studied coagulation in *Anopheles* larvae by applying techniques developed to study clotting in *Drosophila*. To our surprise, we could not draw out strands from coagulating mosquito larval hemolymph as with *Drosophila* [7], suggesting that the mosquito clot is much more delicate. We used more sensitive techniques to demonstrate the formation of a clot in *Anopheles* larvae, and we used pullout [8] to identify phenoloxidase (PO) and apolipophorin-I as candidate clotting factors in these mosquitoes. Electron microscopy of negatively stained clot preparations revealed the *Anopheles* clot is at least partly composed of paracrystalline sheets. Taken together, our data further suggest that these sheets are produced by the assembly of lipophorin particles in a process dependent on PO. Further work will be required to identify additional clotting factors, demonstrate how clot formation is activated, and eluciate the role of coagulation in immune defense in mosquito larvae.

2. Materials and methods

2.1. Mosquito larvae

A. gambiae G3 larvae were raised in standard conditions [15]. Fourth instar larvae were used in all experiments because they are large and close in size to third instar *Drosophila* larvae (Fig. 1A).

2.2. Light microscopy of clot preparations

Five *Drosophila* or *Anopheles* larvae were bled by ripping open with fine forceps into 10 μl 0.1 × PBS pH 8.0 over a copper SEM grid and incubated in a humid chamber 30 min RT. The grids were washed twice in 10 μl drops of 0.1 × PBS, 2 × in 10 μl 0.5% IGEPAL (Sigma) in 0.1 × PBS, and twice in 0.1 × PBS. The clot was then stained with 10 μl 0.1 × PBS containing 10 μg/ml lectin (either HPL-FITC or PNA-FITC) for at least 20 min, and visualized in a Zeiss Axioplan 2 microscope.

2.3. Bead aggregation

Bead aggregation was performed essentially as described in [8]. Tosylactivated Dynabeads (M-280, Dynal) were washed and blocked overnight with 0.2 M Tris (pH 8.5) according to manufacturer's instructions, then washed and resuspended in 0.1 × PBS pH 8.0. For bead aggregation experiments, both *Drosophila* and *Anopheles*, 5 larvae were bled onto 10 μl of beads.

2.4. Pullout

Pullout was performed as described in [8]. Briefly, fourth instar *Anopheles* larvae were rinsed in dH_2O and opened with fine antimagnetic scissors under the surface of 50 μl beads held in a watch glass. The larvae were swirled in the buffer during bleeding to maximize contact of clotting factors with the beads. The solution and the beads were then transferred to a microcentrifuge tube, and the watch glass was washed with an additional 100 μl of buffer, which was added to the tube. Tubes were put in a magnetic holder to attract the beads to the side of the tube, and the solution was removed by pipette. Beads were washed (i) 3 × in 0.1 × PBS, (ii) 3 × in 0.1 × PBS, 0.5% IGEPAL (Nonidet 40) detergent, and Roche protease inhibitor, and (iii) 3 × in 0.1 × PBS. The beads were then resuspended in 16 μl 0.1 × PBS + 5 μl gel loading buffer, heated to 64 °C 10 min, and then stored at −20 °C.

2.5. Transmission electron microscopy (TEM)

Five third instar *Drosophila, or* 10 fourth instar *Anopheles* larvae were bled into 10 μl 0.1 × PBS (with, or without PTU) on top of glow-discharged carbon-coated grids and incubated for 20 min at RT in humid chambers. Grids were then washed twice in 0.1 × PBS, and stained in 1% uranylacetate in 0.1 × PBS for 1 min. The samples were examined in a Philips CM120 Biotwin TEM operating at 100 kV. Images were captured using a retractable CCD camera.