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Major chorion proteins and their crosslinking during chorion hardening in *Aedes aegypti* mosquitoes

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Abstract

The chorion of *Aedes aegypti* eggs undergoes a hardening process following oviposition and individual chorion proteins become insoluble thereafter. Our previous studies determined that peroxidase-catalyzed chorion protein crosslinking and phenoloxidase-mediated chorion melanization are primarily responsible for the formation of a hardened, desiccation resistant chorion in *A. aegypti* eggs. To gain further understanding of peroxidase- and phenoloxidase-catalyzed biochemical processes during chorion hardening, we analyzed chorion proteins, identified three low molecular weight major endochorion proteins that together constituted more than 70% of the total amount of endochorion proteins, and assessed their insolubilization in relation to phenoloxidase- and peroxidase-catalyzed reactions under different conditions. Our data suggest that the three low molecular weight endochorion proteins undergo disulfide bond crosslinking prior to oviposition in *A. aegypti* eggs, and that they undergo further crosslinking through dityrosine or trityrosine formation by peroxidase-catalyzed reactions. Our data suggest that chorion peroxidase is primarily responsible for the irreversible insolubilization of the three major endochorion proteins after oviposition. The molecular mechanisms of chorion hardening are also discussed.

Keywords: *Aedes aegypti*; Chorion proteins; Chorion hardening; Peroxidase

1. Introduction

Although the chorion of mosquito eggs is a densely packed proteinaceous structure (Chapman, 1998), it is susceptible to desiccation and unresistant to detergents or reducing agents. For example, when oviposited eggs were moved to an extremely dry environment immediately following oviposition, they rapidly dehydrated. Essentially all of the chorion proteins were solubilized when dissected mature eggs were treated in a solution containing SDS and a strong reducing agent. However, in a moist environment, the chorion turns black, becomes highly resistant to desiccation within 2 h after oviposition, a process that has been termed chorion hardening. Proteins are the major components in the chorion and they become insoluble after chorion hardening; consequently, chorion hardening likely is due primarily to structural modifications of individual chorion proteins, leading to their insolubilization. Our previous studies have demonstrated that both chorion peroxidase (CPO)- and phenoloxidase-catalyzed reactions are critical for the formation of an insoluble, desiccation-resistant chorion in *Aedes aegypti* (Li and Christensen, 1993; Li et al., 1996). Conceivably, some chorion proteins may serve directly or indirectly as substrates for CPO and chorion phenoloxidase during chorion hardening. To understand the biochemical processes and mechanisms leading to chorion hardening, it is essential to know the identity of individual chorion proteins and their structural modification by CPO or chorion phenoloxidase during chorion hardening.

Ultrastructural studies revealed that there are two recognizable layers in the mosquito chorion, termed endochorion and exochorion, respectively. In mosquitoes, the endochorion is a homogeneous electron-dense layer and the exochorion consists of a lamellar layer with

Abbreviations: CPO, Chorion peroxidase; DETC, Diethyldithiocarbamic acid; ProPO, Prophenoloxidase

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protruding tubercles or a fibrilar network (Monnerat et al., 1999; Soumar and Ndiaye, 2005; Valle et al., 1999). These ultrastructural data suggest that the protein composition and assembly between the two layers might be quite different. Our recent data dealing with the protein profiles of the solubilized chorion proteins revealed that there are three low molecular weight major chorion proteins that are present in the endochorion and that constitute more than 70% of the total amount of endochorion proteins in *A. aegypti*. These major chorion proteins undergo disulfide bond crosslinking prior to oviposition, which is reversible in the presence of a strong reducing agent, and undergo further crosslinking reactions after oviposition, which leads to their irreversible insolubilization. The high abundance of the three low molecular weight major endochorion proteins makes them ideal targets for following the process of insolubilization. This study concerns the identification of these three major endochorion proteins and an assessment of the biochemical reactions resulting in their irreversible insolubilization.

2. Materials and methods

2.1. Materials

CHAPS, diethyldithiocarbamic acid (DETC), dithiothreitol, 2,5-dihydroxylbenzoic acid, phenyl methyl sulfonyl fluoride, sodium deoxycholate, trifluoroacetic acid, chymotrypsin and proteinase K were purchased from Sigma-Aldrich (St. Louis, MO, USA). Triton X100 and HPLC-grade acetonitrile was from Fisher (Pittsburgh, PA, USA). Modified sequence-grade trypsin was from Promega (Madison, WI, USA). C18 cartridge was from Alltech (Deerfield, IL, USA). ZipTip C18 was from Millipore (Bedford, MA, USA). All buffers were prepared with fresh Milli-Q water.

2.2. Egg collection

A. aegypti mosquitoes used in this study were reared according to methods described by Christensen et al. (1984). Adult females were blood-fed on anesthetized guinea pigs. Under the applied rearing conditions, eggs became mature 3 days after taking a blood meal.

Mosquito eggs were collected either through dissecting female mosquitoes at 72 h after a blood meal or by providing a filter paper saturated with different buffers as substrate for mosquitoes to lay eggs. To collect eggs through dissection, female mosquitoes were first cold anesthetized on ice and eggs were dissected into cold insect Ringer's solution (130 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 10 mM HEPES, pH 7.0). After gently washing with the same cold Ringer, the eggs were stored at −80 °C before use.

To assess the role peroxidase and phenoloxidase play in chorion hardening, filter papers, saturated with 10 mM DETC (a phenoloxidase/peroxidase inhibitor) or 10 mM Na_2EDTA (an inhibitor of metallic enzymes) in Ringer's solution, were supplied as substrates for female mosquitoes to lay eggs. Filter paper saturated with Ringer's solution served as a control. After 30 min (when the chorion remains white) and 2 or 3 h (when the chorion turns complete black) incubation at 26 °C, the eggs on filter papers were rapidly collected into Ringer's solution and frozen at −80 °C.

2.3. Chorion isolation and solubilization

Chorion isolation and solubilization were based on described methods in literature (Powell et al., 1986; Trougakos and Margaritis, 1998). Two hundred individual eggs were placed into 5 ml of ice-cold distilled water containing 1% Triton X100, and 1 mM PMSF. Eggs were first broken mechanically and then sonicated for 1 min to release oocytic materials. Chorion was collected by sedimentation on ice for 15 min and solubilized oocytic materials were poured off. The chorion sediments were washed three more times with the same solution. The isolated chorion sediments, free from oocytic components under microscopic examination, were solubilized in 0.1 ml Leammli buffer (0.125 mM Tris, pH 6.8, 2% SDS, 10% glycerol and 0.1 M DTT) at 70 °C for 30 min. After centrifugation at 20,000*g* for 10 min, the solubilized proteins were subjected to SDS–PAGE.

2.4. SDS–PAGE, electro-elution and in-gel digestion

The solubilized proteins were separated by SDS–PAGE with 12% acrylamide gel (Laemmli, 1970). After electrophoresis, the gel with separated proteins was stained with Coomassie blue. The protein bands on the gel were excised, and then eluted at 50 mA for 2 h using an electroelution device (Millipore, Bedford, MA, USA). The eluted protein was concentrated using an Amicon YM-10 centrifugal filter (Millipore). Aliquots of the samples were applied to MALDI/TOF/MS to determine the molecular mass of the intact proteins.

After reduction and alkylation, the aliquots of the protein samples were digested with trypsin (0.01 μg/μl) or chymotrypsin (0.01 μg/μl) in 50 mM Tris–HCl (pH 8.0) at 37 °C for 16 h, or digested with proteinase K (0.01 μg/μl) in 0.2 M Na_2CO_3 (pH 11.0) at 37 °C for 5 h (Wu et al., 2003). The samples were then applied to LC/MS/MS for de novo sequencing.

2.5. MALDI/TOF/MS and LC/ESI/MS/MS

MALDI spectra were acquired on a Voyager-DE STR (Applied Biosystems, Norwalk, CT, USA) in positive ion mode with a delayed extraction ion source. A capillary LC interfaced through a nanospray ESI source with a Q-TOF micro™ mass spectrometer (Waters Micromass, Manchester, UK) was used for LC/MS/MS. Peptide separation was achieved through gradient elution with mobile phase