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Molecular characterization of *Rhodnius prolixus*' embryonic cuticle

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In memoriam of our colleague Dr. Alexandre Peixoto.

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ABSTRACT

The embryonic cuticle (EC) of *Rhodnius prolixus* envelopes the entire body of the embryo during hatching and provides physical protection, allowing the embryo to pass through a narrow chorionic border. Most of the knowledge about the EC of insects is derived from studies on ultrastructure and secretion processes during embryonic development, and little is known about the molecular composition of this structure. We performed a comprehensive molecular characterization of the major components extracted from the EC of *R. prolixus*, and we discuss the role of the different molecules that were identified during the eclosion process. The results showed that, similar to the post-embryonic cuticles of insects, the EC of *R. prolixus* is primarily composed of carbohydrates (57%), lipids (19%), and proteins (8%). Considering only the carbohydrates, chitin is by far the major component (approximately 70%), and it is found primarily along the body of the EC. It is scarce or absent in its prolongations, which are composed of glycosaminoglycans. In addition to chitin, we also identified amino (15%), neutral (12%) and acidic (3%) carbohydrates in the EC of *R. prolixus*. In addition carbohydrates, we also identified neutral lipids (64.12%) and phospholipids (35.88%). Proteomic analysis detected 68 proteins (55 were identified and 13 are hypothetical proteins) using the sequences in the *R. prolixus* genome (http://www.vectorbase.org). Among these proteins, 8 out of 15 are associated with cuticle metabolism. These proteins are unequivocally cuticle proteins, and they have been described in other insects. Approximately 35% of the total proteins identified were classified as having a structural function. Chitin-binding protein, amino peptidase, amino acid oxidase, oxidoreductase, catalase and peroxidase are all proteins associated with cuticle metabolism. Proteins known to be cuticle constituents may be related to the function of the EC in assisting the insect during eclosion. To our knowledge, this is the first study to describe the global molecular composition of an EC in insects.

1. Introduction

In recent years, possibly due to the availability of the *Rhodnius prolixus* genome (http://www.vectorbase.org), an increasing number of studies at the molecular level have performed to understand the biology of this species. *R. prolixus* is one of the main vectors of Chagas disease in Latin America (Schofield, 2000). This species produces approximately 40 eggs per female after a single blood meal (Atella et al., 2005). Oviposition occurs in the environment, and embryonic development lasts approximately 15 days and varies with ambient temperature (Kelly and Huebner, 1989; Melllanby, 1935; Wigglesworth, 1972). The main hurdle faced by *R. prolixus* nymphs during eclosion is exiting the chorion through the narrow chorionic border orifice. The EC envelopes the entire body of the hatching insect, providing physical protection and serving as a conducting channel as the insect passes through the chorionic border (Wigglesworth, 1972). The presence of an EC has been reported in several insect orders (Konopova and Zrzavy, 2005). Moussian et al. (2006) showed that cuticle differentiation occurs in three phases during *Drosophila melanogaster* embryogenesis that is genetic controlled (Payre,

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2004). The layers that are established partially simultaneously in the first stage thicken in the second stage. After the secretion of materials ceases in the third phase, the chitin laminae acquires its final orientation and the epicuticle darkens. The EC is described as a highly elastic, transparent layer that separates the embryo from the yolk membrane during embryogenesis (Louvet, 1974), but its biology and formation have not been thoroughly investigated (Fewkes, 1968; Konopova and Zrzavy, 2005; Provine, 1976; Wigglesworth, 1972). The knowledge of embryonic cuticles (EC) is based on data on the ultrastructure and the secretion processes during the embryonic development of insects. Conversely, studies on post-embryonic cuticles are more comprehensive and include more detailed data on their structure, chemical composition, morphogenesis and the protein products of the cuticular genes (Dombrovsky et al., 2003; Juarez and Fernandez, 2007; Wigglesworth, 1972). The post-embryonic cuticle of insects gives them shape, flexibility, strength, freedom of movement, defense, and protection; assists in homeostasis; protects against excessive moisture and desiccation; and prevents penetration by microorganisms. The success of these functions is directly related to the biochemical composition of the cuticle, and there is a direct correlation between its lipid composition and its permeability, resistance, and malleability. In triatomines, post-embryonic cuticles are primarily composed of neutral lipids, and these cuticles also have high concentrations of triacylglycerol, diacylglycerol, fatty acids, and hydrocarbons. Phospholipids are primarily composed of phosphatidylethanolamine and phosphatidylcholine (Gibbs, 2002; Juarez and Fernandez, 2007). The rigidity of the exoskeleton is conferred by sugar polymers such as chitin (Ostrowski et al., 2002; Richards, 1978). Chitin is a β-glycoside biopolymer composed of units of β-(1-4)-N-acetyl-D-glucosamine, which is the major carbohydrate that is found in the post-embryonic cuticle of insects. In general, chitin corresponds to approximately 20–50% of the animal's dry weight, and variations in the chitin composition of insect cuticles are found among different species, stages, and body parts in the same individual and between sexes in the same species (Moussian et al., 2006; Okot-Kotber et al., 1994; Ostrowski et al., 2002; Richards, 1978; Wigglesworth, 1972).

Proteins are the group of macromolecules with the highest number and diversity in insect cuticles, and they are largely responsible for the most significant differences that are found in cuticles. Proteins associated with chitin confer strength and rigidity to cuticles, in addition to flexibility and freedom of movement, and they are essential in adaptation, response, and regulatory processes (Kramer and Muthukrishnan, 2005; Merzendorfer and Zimoch, 2003).

Some of the information about insect cuticular proteins (CuPs) comes from sequencing of the N-terminal region of a few proteins extracted from insect cuticles (Hojrup et al., 1986), proteomic analysis (He et al., 2007) and protein sequences derived from the transcription of cDNAs (Asano et al., 2013; Dombrovsky et al., 2003), which were identified based on their similarities to proteins extracted from post-embryonic cuticles (Dombrovsky et al., 2007; Togawa et al., 2008; Willis, 2010).

Although more information is available on post-embryonic than ECs, much remains to be discovered and understood in both cases. Possibly due to the difficulty in obtaining enough material from ECs, little is known about their molecular composition. Taking advantage of our access to a large colony of *R. prolixus*, we were able to directly extract lipids, carbohydrates, and proteins from a vast quantity of EC for analysis. Lipids and carbohydrates were analyzed by standard methods such as TLC, FTIR spectroscopy, HPLC, and fluorescence microscopy, and proteins were analyzed by proteomic technology.

Insect cuticles are comprised of various materials including a mixture of proteins, lipids, carbohydrates, and other minor components. The mechanical properties of the cuticle depend largely on its composition, as well as on the number of interactions that occur among its constituents. Thus, a variety of mechanical characteristics can be obtained, allowing the multimolecular structure to fit the needs of a particular physiological function. In *Rhodnius*, the composition of an extensible cuticle has been studied by Hillerton (1978).

Here, we performed a comprehensive molecular characterization of the major components extracted from the EC of *R. prolixus*, and we compare our results with published data on post-embryonic cuticles. We then discuss the importance of these macromolecules on the eclosion process of first-instars nymphs.

2. Materials and methods

2.1. Reagents

All reagents were analytical grade and were acquired from Sigma Chemical Co. (St Louis, MO, USA), Merck (Rio de Janeiro, Brazil), Reagen (Rio de Janeiro, Brazil) or Vetec (Rio de Janeiro, Brazil) and Invitrogen (São Paulo, Brazil). All solutions were prepared using double-distilled water from a Milli-Q system with ion-exchange resins (Millipore Corp, Bedford, MA, USA).

2.2. Preparation of Rhodnius prolixus embryonic cuticles (EC)

R. prolixus females were reared and maintained at 28 °C and 70–80% relative humidity, and they were fed rabbit blood in the Laboratory of Insect Biochemistry, Federal University of Rio de Janeiro, Brazil. Fertilized eggs were collected hourly, handled in a sterile environment, and stored in sterile vials under the same climatic conditions as the colony. Embryonic cuticles (EC) were collected after eclosion of first-instars nymphs using sterile sieves, spatulas, and tweezers. All animal care and experimental protocols were conducted following the guidelines of the institutional care and use committee (Committee for Evaluation of Animal Use for Research from the Federal University of Rio de Janeiro, CAUAP-UFRJ, Brazil). The protocols were approved by CAUAP-UFRJ under registry #IBqM001.

2.3. Dry weight composition of embryonic cuticle of R. prolixus

To estimate the dry weight of different components of *R. prolixus*' EC, 3 mg of EC was dehydrated and weighed (total). Next, the material was placed in conical tubes, and total lipid extraction was initiated by adding 4 mL of methanol:chloroform:double-distilled water solution (2:1:1 v/v), followed by intermittent agitation every 5 min for 2 h. The samples were then left standing for 12 h at 4 °C. The mixture was centrifuged for 15 min at 1500 × g, and the hydrophobic phase was saved. Then, 1 mL of chloroform was added to the remaining material, and the procedure was repeated twice. The hydrophobic phases were pooled, dried in polypropylene tubes, and weighed to measure the amount of lipids extracted (lipid fraction). The remaining fraction was dried and weighed again before treatment with papain (1 mg/mL in 5 mM sodium acetate, pH 5.0, containing 5 mM EDTA and 5 mM cysteine) for 24 h at 65 °C to remove proteins. The remaining material was washed by centrifugation for at 1550 × g for 15 min, and the supernatant (A) and precipitate (B) were saved. Ethanol (1:1 v/v) was added to the supernatant (A) to precipitate soluble carbohydrates for 24 h at 4 °C, and then they were separated by centrifugation at 1500 × g for 15 min. Hexane and H_2O (1:1 v/v) were added to the precipitate (B) to remove other hydrophobic

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