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Proteomic analysis of the crayfish gastrolith chitinous extracellular matrix reveals putative protein complexes and a central role for GAP 65

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ABSTRACT

Chitin is a major component of arthropod cuticles, where it forms a three-dimensional network that constitutes the scaffold upon which cuticles form. The chitin fibers that form this network are closely associated with specific structural proteins, while the cuticular matrix contains many additional structural, enzymatic and other proteins. We study the crayfish gastrolith as a simple model for the assembly of calcified cuticular structures, with particular focus on the proteins involved in this process. The present study integrates a gastrolith-forming epithelium transcriptomic library with data from mass spectrometry analysis of proteins extracted from the gastrolith matrix to obtain a near-complete picture of gastrolith protein content. Using native protein separation we identified 24 matrix proteins, of which 14 are novel. Further analysis led to discovery of three putative protein complexes, all containing GAP 65 the most abundant gastrolith structural protein. Using immunological methods we further studied the role of GAP 65 in the gastrolith matrix and forming epithelium, as well as in the newly identified protein complexes. We propose that gastrolith matrix construction is a sequential process in which protein complexes are dynamically assembled and disassembled around GAP 65, thus changing their functional properties to perform each step in the construction process.

Biological significance: The scientific interest on which this study is based arises from three main features of gastroliths: (1) Gastroliths possess partial analogy to cuticles both in structural and molecular properties, and may be regarded, with the appropriate reservations (see Introduction), as simple models for cuticle assembly. At the same time, gastroliths are terminally assembled during a well-defined period, which can be controlled in the laboratory, making them significantly easier to study than cuticles. (2) Gastroliths, like the crayfish exoskeleton, contain stable amorphous calcium carbonate (ACC) rather than crystalline calcite. The biological mechanism for the stabilization of a naturally unstable, but at the same time biologically highly available, calcium carbonate polymorph is of great interest from the pharmaceutical point of view. (3) The gastrolith organic matrix is based on a highly structured chitin network that interacts with a variety of substances. This biologically manipulated, biodegradable structure is in itself of biotechnological and pharmaceutical potential. A growing body of evidence indicates that proteins play central roles in all above aspects of gastrolith construction.

This study offers the first comprehensive screening of gastrolith proteins, and we believe that the analysis presented in this work can not only help reveal basic biological questions regarding assembly of mineralized and non-mineralized cuticular structures, but may also serve as basis for applied research in the fields of agriculture (e.g. cuticle-based pest management), health (e.g. bioavailable calcium supplements and biodegradable drug carriers) and materials science (e.g. non-toxic scaffolds for water purification).

1. Introduction

Chitin, the second-most abundant polysaccharide in nature after cellulose, is a major component of arthropod cuticles, where it is assembled into three-dimensional networks that serve as light but mechanically strong scaffolds [1,2]. While it is known that cuticular chitin is assembled by the enzyme chitin synthetase [3], the specific mechanism by which chitin is produced remains unknown. Available evidence suggests that chitin is synthesized by membrane-bound clusters of chitin synthase catalyzing the formation of linear chains consisting of β-(1 → 4) linked *N*-acetyl-D-glucosamine (GlcNAc)

Abbreviations: ACC, amorphous calcium carbonate; CqCBP, *C. quadricarinatus* chitin-binding protein; CqCc1, *C. quadricarinatus* cryptocyanin 1; GFE, gastrolith-forming epithelium; GMPs, gastrolith matrix proteins; LPS-BP, lipopolysaccharide and beta-1,3-glucan-binding protein; LEA, late embryogenesis abundant; NGS, next-generation sequencing.

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subunits. These chains are then translocated across the plasma membrane to the extracellular matrix, where they assemble into crystalline microfibrils via interchain hydrogen bonding. Subsequent association with cuticular proteins and the formation of an ordered network of cross-linked fibers ensue [1,4–7]. Cuticular proteins are long recognized as serving many different roles, including chemical modification of the chitin microfibrils [8], remodeling of the chitin network [9], immunological defense [10], pigmentation [11–14] and cross-linking of chitin microfibrils in a process called sclerotization [15,16].

In the exoskeletons of most crustaceans, cuticular sclerotization is followed by the deposition of minerals, namely calcium carbonate [7,17], as an additional hardening factor. Calcium carbonate is deposited either as the crystalline form of calcite, or as stable ACC [7]. ACC is a naturally unstable form of calcium carbonate, and substantial evidence has shown that in the crustacean exoskeleton, this compound is stabilized through the involvement of proteins and/or peptides [18,19] as well as organic and inorganic molecules and ions [20–23].

Exoskeleton formation is a cyclical process performed periodically as the animal grows and molts. During each molt event, there is formation of a new cuticular chitin–protein structure, which in crustaceans also corresponds with a calcification process. To generate a new exoskeleton, crayfish rely on gastroliths, cuticle-like structures that serve as temporary calcium storage organs, and assist in the fast hardening of the new post-molt cuticle [18,24]. Gastroliths are formed in an enclosed pouch found between the gastrolith-forming epithelium and the cuticular stomach wall [25]. Like the exoskeleton, gastroliths are composed of an organic matrix consisting of chitin and proteins, with precipitated mineral in the specific form of ACC [17]. The resemblance between gastroliths and the exoskeleton can be seen not only in terms of ultrastructure but also in transcript expression and protein content [19,26,27]. On the other hand, gastroliths lack some of the structural complexity of the exoskeleton [17,25,28] and are, therefore, considered as simple models that can be used to study certain aspects of exoskeleton structure and formation [12,29].

In our long-term study of crayfish gastrolith structure, composition and formation [12,17,19,27,29], one of our main goals has been to achieve as complete a picture of proteins participating in gastrolith formation as possible and to study potential interactions between them. So far, three proteins, namely GAMP from the gastroliths of the crayfish *Procambarus clarkii* [30–32], and GAP 65 and GAP 10, from the gastroliths of the crayfish *Cherax quadricarinatus* [19,27], have been identified as being expressed in the gastrolith-forming epithelium and secreted to the gastrolith matrix. All three predicted protein sequences were obtained through the use of mass spectrometric peptide sequencing for directing the synthesis of degenerate primers, followed by PCR amplification and sequencing of the responsible transcripts. In some cases, the process was aided by the generation of cDNA libraries from relevant tissues. A fourth deduced protein, CqCDA1, was identified based on the detection of significant up-regulation of its transcript during pre-molt in the gastrolith-forming epithelium in a microarray analysis of pre-molt versus intermolt transcript levels [26].

In 2012, we suggested a structural model assigning specific roles for these four proteins and predicting the participation of additional, yet to be identified, proteins in gastrolith formation [12]. Our model was based on the division of gastrolith proteins into three functional groups: (1) proteins that are exclusively involved in construction of the chitinous matrix, (2) proteins that are directly involved in the precipitation of the ACC and that have no direct association with chitin, and (3) proteins that participate in both processes (i.e. multi-task proteins). The above-mentioned gastrolith proteins were assigned to the appropriate group based on sequence analysis and other published empirical data. In addition to assigning potential functions, we suggested the existence of protein–protein interactions involved in the formation of gastrolith protein complexes necessary for attachment of non-chitin-binding proteins to the organic matrix, as well as for other purposes. However, additional information on gene expression in the gastrolith-forming epithelium and on protein sequences from the gastrolith matrix to identify such interactions was not available at the time.

In recent years, protein biochemistry studies have reached the level of high throughput largely due to advances made in tandem mass spectrometry technology [33,34]. Next-generation sequencing (NGS)-generated transcriptomic libraries can be used as databases for such MS-based studies [29], especially when studying a biological system responsible for the assembly of a well-defined construct in a specialized tissue. The crayfish gastrolith offers an ideal platform for applying this strategy, in addition to being a good model for studying exoskeleton formation. Accordingly, we recently employed the NGS method of 454-sequencing to obtain a transcriptome of *C. quadricarinatus* epithelial tissues [29]. The RNA samples were extracted from the gastrolith-forming and sub-cuticle epithelia at four molt stages, i.e. intermolt, early pre-molt, late pre-molt and post-molt.

In the present study, we have made use of this comprehensive epithelial library to complement protein biochemistry efforts aimed at cataloging those proteins incorporated into *C. quadricarinatus* gastroliths. In doing so, several novel proteins were identified. In addition, we present evidence for the existence of three dominant protein complexes within the gastrolith, and identify the putative protein composition of each suspected complex. We further found that GAP 65 is present in each complex. On the basis of this last finding together with evidence from immunostaining experiments detecting GAP 65 distribution and localization in the gastrolith-forming epithelium (GFE) and gastrolith matrix, we propose that GAP 65 serves as a central protein in the identified protein complexes.

2. Materials and methods

2.1. Animals and molt

C. quadricarinatus males were grown in artificial ponds at Ben-Gurion University of the Negev, Beer-Sheva, Israel, under conditions described in Shechter et al. [17]. Inter-molt crayfish were held in individual cages and endocrinologically induced to enter pre-molt through removal of the X organ-sinus gland (XO-SG) complex or repeated ecdysone injection. Progression of the molt cycle was monitored daily by measuring the gastrolith molt mineralization index (MMI), as described by Shechter et al. [35]. For all dissection procedures, crayfish were placed on ice for 10–15 min, until anesthetized.

2.2. Immunodetection of GAP 65 in the gastrolith matrix and gastrolith-forming epithelium by fluorescence microscopy

For immunohistochemistry, whole gastrolith pouches were submerged in a decalcifying fixative containing 7% EDTA and 0.2% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS). Alternatively, an isolated GFE was submerged in 4% formaldehyde in PBS, dehydrated in ethanol and embedded in paraffin. Paraffin sections (5 μm-thick) were deparaffinized, rehydrated, incubated in 0.5 M citrate buffer, pH 6.0 (30 min at 95 °C) for antigen retrieval and washed in 0.01 M PBS, pH 7.4. Blocking with 2% normal goat serum, 0.1% Triton X-100, and 0.05% Tween 20 in PBS was performed for 1 h at room temperature (RT), followed by incubation with rabbit polyclonal anti-GAP 65 antisera as primary antibodies (1:1000, v/v). The slides were washed in PBS and incubated with secondary goat anti-rabbit FITC conjugated antibodies (1:250 in PBS containing 0.2% fish skin gelatin) for 1 h at RT. After PBS washes, the slides were mounted (DAPI 1:1000 in PBS and 50% glycerol) and imaged using a fluorescence microscope.

2.3. GFE ultrastructure analysis by transmission electron microscopy (TEM)

GFEs were excised from late pre-molt animals and fixed in 2% glutaraldehyde in 0.2 M phosphate buffer (PB) pH 6.8, at RT for 1 h. The specimens were then fixed on ice for 1 h in a second fixative comprising of