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On the involvement of proteins in the assembly of the crayfish gastrolith extracellular matrix

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It has been suggested that gastroliths are a good model for the study of biomineralization and extracellular organic matrix assembly, since they can serve as simplified cases of more complex mineralized biological structures, such as cuticles, bone, and teeth. Proteins are known to be key players in biomineralized chitinous matrices in general and in gastrolith assembly in particular. Here, we present a structural model that is based on the integration into the gastrolith assembly of the gastrolith proteins identified thus far. The model allows the assignment of these proteins to the different aspects of gastrolith construction, based on their characteristics. The model also predicts the existence of several additional proteins yet to be identified.

Keywords: gastrolith proteins; extracellular matrix; amorphous calcium carbonate; biomineralization

Introduction

Biomineralization and its supporting extracellular processes are common phenomena throughout the animal kingdom. The processes are essential to the building of all skeletal components comprising the main biogenic calcium reservoir for living species that provide support, protection, and scaffolding for soft tissue.

Arthropods, the largest group of species, have rigid external skeletons, known as exoskeletons, that are composed of an organic matrix consisting of α-chitin microfibrils and proteins (Blackwell and Weih 1980; Lowenstam and Weiner 1989). These chitin–protein fibers form a network of chitin–protein layers that are helicoidally stacked into a twisted plywood pattern (Bouligand 1972; Raabe et al. 2005b). The rigidity of the exoskeleton is achieved through the enzymatic oxidation of phenols or catechols, which then interact with the cuticular proteins and chitin to crosslink and harden them in a process known as sclerotization (Kuballa and Elizur 2008). In most crustacean species, the exoskeletons are further hardened by the deposition of minerals, mainly calcium carbonate (Lowenstam and Weiner 1989). The calcium carbonate is deposited either as the crystalline form of calcite, and/or as stable amorphous calcium carbonate (ACC) (Lowenstam and Weiner 1989). ACC is naturally an unstable form of calcium carbonate, but it is believed to be stabilized in biological systems by macromolecules and/or specific ions (Aizenberg et al. 1996). Although there is evidence for ACC stabilization by Mg ions (Loste et al. 2003) or by phosphate groups (Clarkson et al. 1992), a growing number of studies have shown that ACC stabilization requires the involvement of proteins and/or peptides (Luquet and Marin 2004; Shechter et al. 2008b).

In crustaceans, as in all arthropods, the exoskeleton is periodically shed and rebuilt – in a process known as molting, for purposes of growth, regeneration, metamorphosis, and reproduction. The molt cycle in crustaceans is divided into four major stages (Drach and Tchernigovtzeff 1967; Skinner 1985; Aiken and Waddy 1987; Chang 1991; Chang 1993): (1) premolt, during which molt preparatory events take place, such as the formation of a new cuticle, separation of the old cuticle from the underlying subepidermal tissue in a process known as apolysis, and some degradation of the old cuticle; (2) ecdysis, the shedding event; (3) postmolt, during which parts of the new cuticle are synthesized and calcium is mobilized and deposited in the new cuticle; and (4) intermolt, during which all processes related to the previous molt have been completed and the cuticle is fully formed and maximally calcified.

The molting process is accompanied by a significant loss of cuticular calcium, which the animal is required to quickly regain during postmolt so as to enable it to deal with the threat of predation and to start feeding. Most crustacean species live in marine environments where calcium is readily available for uptake, but freshwater and terrestrial species require temporary storage solutions to provide them with at

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least some of the calcium they require for full calcification of the new exoskeleton (Greenaway 1985). It should be noted that calcium storage also takes place in some marine crustaceans. There are several strategies for calcium storage in crustaceans, and they have all been reviewed in a comprehensive manner by Luquet and Marin (2004). Of particular relevance to the current review is the overview given by Luquet and Marin (2004) of protein involvement in the formation of the organic matrices of calcified structures, both exoskeleton and storage organs, in crustaceans.

In crayfish (Travis 1963a, 1963b), lobsters, and some land crabs (Luquet and Marin 2004), calcium carbonate storage organs, such as gastroliths, are formed on both sides of the cardiac stomach wall. The gastroliths are formed during premolt. They reach their peak size shortly before ecdysis, when they collapse into the stomach. During postmolt, the gastroliths are completely digested in the stomach, and the calcium is transported through the hemolymph for deposition in the new exoskeleton. The major mineral phase in the gastroliths is stable ACC (Travis 1963b; Shechter et al. 2008a).

At the time of the review of Luquet and Marin (2004), there was only one known gastrolith protein (GAP), gastrolith matrix protein (GAMP) – which had been identified and characterized in the crayfish *Procambarus clarkii* (Ishii et al. 1996, 1998; Tsutsui et al. 1999). In this article, we focus on crayfish gastroliths as a relatively simple model for the study of biomineralization within extracellular organic matrices. We review the latest progress in the ongoing process of identification and characterization of GAPs and suggest a hypothetical structural model that allows the assignment of specific roles to known proteins and the prediction of additional proteins yet to be identified.

The gastrolith structure

Gastroliths are deposited by the gastrolith-forming tissue, a columnar epithelium; they are formed in the lumen between the epithelium and the stomach wall (Figure 1, *Cherax quadricarinatus*). The main functions of the epithelium are the transport of hemolymph calcium to the gastrolith and the synthesis of the gastrolith organic matrix (Ueno 1980). Like the exoskeleton, gastroliths are made of a chitin–protein organic matrix in which calcium carbonate is deposited (Roer and Dillaman 1984; Luquet and Marin 2004).

One of the first detailed descriptions of gastroliths was given by T.H. Huxley (1879) in his book entitled "The Crayfish: An Introduction to the Study of Zoology". Huxley described the gastrolith, also known as "crab's eyes", as "composed of thin superimposed layers, of which the inner are parallel with the flat inner surface, while the outer becomes gradually concentric with the outer surface." This layered structure observed by Huxley is, in fact, the arrangement of the chitin–protein layers that can be seen by light microscopy (Figure 1).

Over the years, a few additional and more thorough descriptions of the lamellar arrangement of the gastroliths have been published. Travis (1960, 1963a), in her extensive study of the gastrolith tissue of the crayfish Orconectes virilis Hagen using transmission electron microscopy and light microscopy, described the gastrolith matrix as being constructed of "fibrous lamellae." However, the first scanning electron microscopy (SEM) study of gastroliths – in this case, the surfaces of fractured gastroliths (Shechter et al. 2008a) – revealed "onion-like structures of concentric mineral layers separated by lower density layers that probably contain more organic material." These layers also can be seen in the SEM micrograph of a C. quadricarinatus gastrolith shown in Figure 2(A). A close look at areas in the gastrolith structure in which the layers are more loosely packed shows thin chitin lamellas about 1 μm thick (Figure 2(B)). These thin lamellas comprise the thicker layers observed in Figure 2(A). In addition, the horizontal chitin lamellas are cross-linked by many vertical chitin strands, indicating that the spatial structure of the gastroliths is determined by the three-dimensional arrangement of its chitinous matrix. This observation coincides with the report of Shechter et al. (Shechter et al. 2008a) that the spatial structure is maintained in demineralized gastroliths.

Decalcification of the gastrolith matrix revealed a three-dimensional chitin network forming the layers within which calcium is deposited for storage (Figure 3). The network is observed to be fairly rigid and organized, with numerous connections and branchings. The chitin networks of crustacean exocuticle and endocuticle have been described as well-ordered planar honeycombs, in which the chitin–protein fibers creating the network are connected in hexagonal arrays (Raabe et al. 2005a). To date, however, the network organization of the gastrolith chitin has not been studied, although it does not seem to be ordered in the fashion of a honeycomb as it is in the exoskeleton.

Electron microscopy studies of calcified gastroliths showed that ACC is precipitated as nano-spherules (Shechter et al. 2008a). Our high magnification electron micrographs reveal a close association between the spherules and the chitin strands (Figure 4(A)). The spherules are not precipitated freely in the hollows formed within the network, but appear to be formed on and around the chitin fibers (Figure 4(B)).

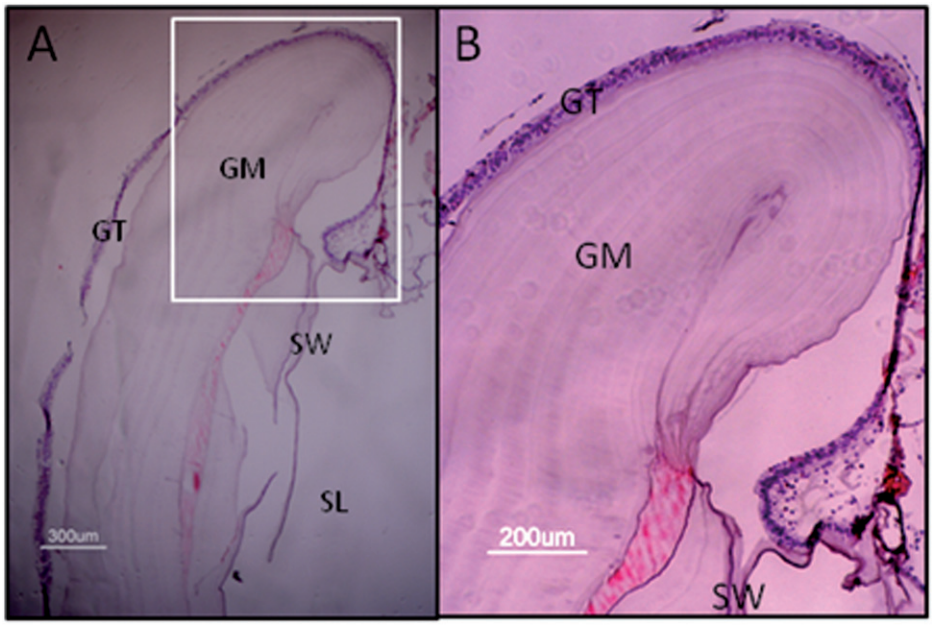

Figure 1. Decalcified gastrolith pouch stained with hematoxylin and eosin. (A) Low-magnification light micrograph of the gastrolith matrix (GM) and tissue (GT), and the stomach wall (SW). The area of the stomach lumen (SL) is indicated. (B) Higher magnification of the boxed area in A showing the order of the layers.

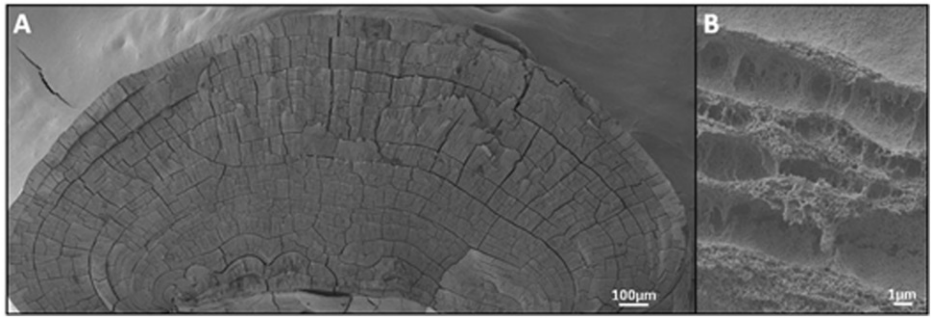

Figure 2. High-resolution SEM (HR-SEM) micrograph of a gastrolith fracture. (A) General view at low magnification. (B) Higher magnification of a loosely packed area in which the thin lamellas are visible.

Identification of gastrolith proteins

Figure 5 illustrates the two-step procedure generally used for the extraction of GAPs, as reported by (Ishii et al. 1998; Shechter et al. 2008b). The first step comprises decalcification of the gastroliths for the extraction of the proteins that are loosely attached to the matrix. These proteins are thus known as soluble proteins (Sol). This step consists of treatment with EGTA or EDTA. It can also be achieved using acidic solutions containing acetic acid or HCl, but this approach might be harmful to the extracted proteins. The second step consists of treatment with denaturing agents, such as 6 M urea or sodium dodecyl sulfate (SDS), for the extraction of proteins that are directly and more strongly bound to the chitinous matrix, probably through hydrogen bonds. These proteins are known as insoluble proteins (Ins). In the case of GAMP, treatments with 6 M urea or SDS alone were not sufficient to extract the protein, and only the application of more aggressive conditions, specifically a combination of SDS and dithiotreitol and heating to 100°C, enabled detachment of the protein from the chitin (Ishii et al. 1998). The need for such harsh extraction conditions led Ishii et al. (1998) to believe that GAMP is strongly bound to the chitin, possibly through covalent bonds. Moreover, when considering that the amount of GAMP extracted from gastroliths was larger than the total of all other extracted proteins, it was suggested by the Nagasawa group that GAMP is the protein referred to as part of the basic chitin–protein fibers comprising the chitinous network (Ishii et al. 1998). The sequence of GAMP does not contain any known domains, but it does contain two unique

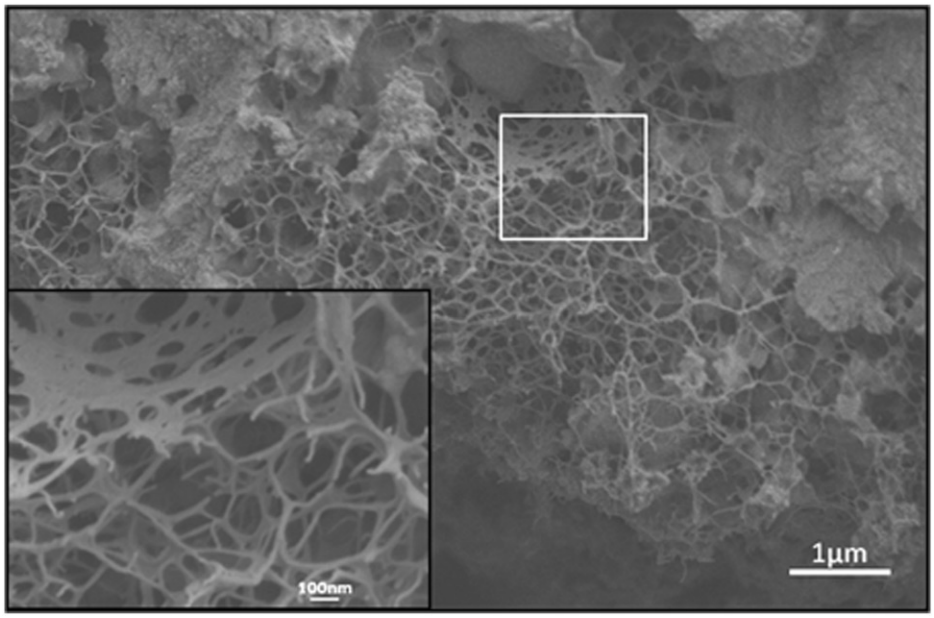

Figure 3. HR-SEM micrograph of a calcium deficient area within a gastrolith. Insert – Higher magnification of the boxed area.

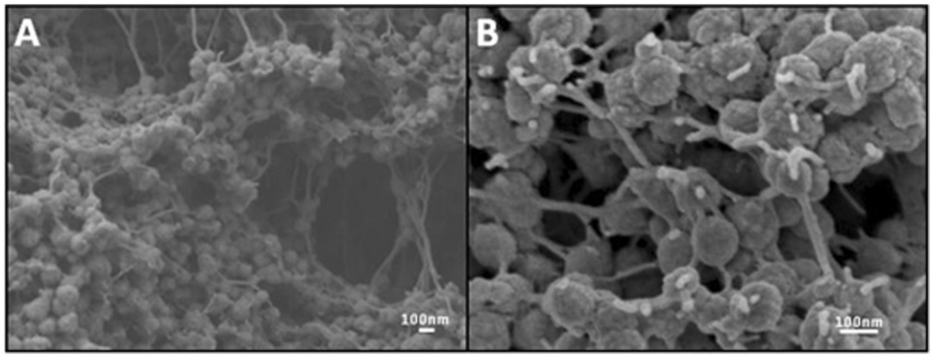

Figure 4. High magnification HR-SEM micrograph views of ACC spherules closely attached to the chitin strands. The magnification in B is higher than in A.

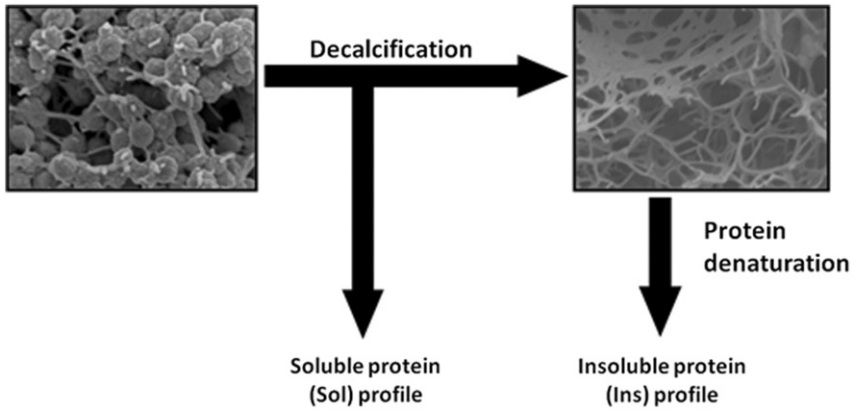

Figure 5. Two step procedure used for extraction of soluble (Sol) and insoluble (Ins) GAPs.

repeated sequences. One of the repeated sequences showed some similarity to that of involucrin, a keratinocyte protein (Ishii et al. 1998). It was also suggested that GAMP is involved in gastrolith calcification, since it inhibits calcium carbonate crystallization in solution (Tsutsui et al. 1999).

At the time that Luquet and Marin (2004) wrote their review on storage strategies and related proteins,

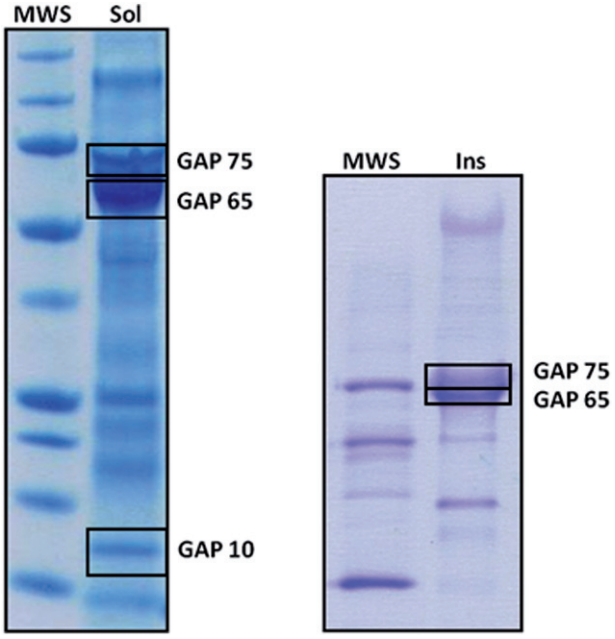

Figure 6. SDS-PAGE separations of *C. quadricarinatus* EGTA extracted (Sol) and 6 M urea extracted (Ins) proteins after Coomassie brilliant blue staining. The bands corresponding to identified GAPs are marked by rectangles. MWS – molecular weight standard.

only GAMP had been identified (Ishii et al. 1996). Since then, three more proteins have been identified, all from the gastroliths of another crayfish, *C. quadricarinatus*. Figure 6 shows the identification of the three proteins according to their migration on SDS-PAGE at apparent approximate molecular masses 10, 65, and 75 kDa; these proteins were thus termed GAPs 10, 65, and 75, respectively (Shechter et al. 2008b; Bentov et al. 2010; Glazer et al. 2010). GAP 65 and GAP 75 were identified in both the Sol and the Ins profiles, while GAP 10 was identified only in the Sol profile. A fourth protein was deduced from a gene identified in a microarray experiment conducted on the basis of a gastrolith-forming tissue transcript library, and designated *C. quadricarinatus* chitin deacetylase 1(CqCDA1) (Yudkovski et al. 2010).

GAP 65 was the most abundant protein found in the *C. quadricarinatus* GAP profile (Shechter et al. 2008b). The protein was identified as a negatively charged glycoprotein. The complete protein sequence was deduced from the fully sequenced transcript, obtained using degenerative primers based on *de novo* peptide sequencing performed on mass spectra (MS) of the trypsinized protein. GAP 65 is predicted to have a signal sequence directing it to the secretion pathway, and three domains – chitin-binding domain 2 (ChtBD2; also known as peritrophin A), low-density lipoprotein receptor class A domain, and polysaccharide deacetylase domain. Based on this prediction, which included two potentially chitin-binding domains – ChtBD2 and polysaccharide deacetylase – it is suggested that GAP 65 is a chitin-binding protein. In addition, the protein sequence has a long C-terminal stretch containing several cysteine residues possibly forming disulfide bridges. The function of this stretch is, however, unknown. The silencing of *GAP 65* transcripts through dsRNA injections to premolt crayfish resulted in the development of gastroliths with morphological deformities both in the general shape and at the nano-scale level, and in a decrease in mineral density. GAP 65 was found to possess a calcium-binding ability in a radioactive calcium-binding assay, and to stabilize synthetic ACC *in vitro*. These data led Shechter et al. (2008b) to the conclusion that GAP 65 has a dual function, both in the formation of the extracellular matrix and in the calcification process of the gastrolith by stabilizing ACC.

GAP 10, the exclusively Sol protein, was also fully sequenced using MS/MS (Glazer et al. 2010). The deduced protein does not contain any known domains, other than a signal sequence, but its amino-acid composition is typical of proteins that are extracted from the calcified extracellular matrixes of invertebrates (Benson et al. 1986; Tsutsui et al. 1999; Watanabe et al. 2000; Inoue et al. 2001, 2004; Pereira-Mouries et al. 2002; Gotliv et al. 2003; Raz et al. 2003; Zhang et al. 2003; Gayathri, et al. 2007; Marie et al. 2007) and ascidians (Aizenberg et al. 2002). GAP 10 was shown to be a phosphorylated and calcium-binding protein. In a microarray experiment, the transcript encoding for GAP 10 was found to be highly up-regulated in the gastrolith-forming tissue during premolt (Yudkovski et al. 2010). Silencing the transcript using dsRNA injected into premolt crayfish, as in the silencing of *GAP 65*, resulted in a prolonged premolt stage and the development of gastroliths with irregularly rough surfaces (Shechter et al. 2008b).

GAP 75, identified by (Bentov et al. 2010) as a doublet band at ~70–75 kDa, is the only researched GAP that has not yet been sequenced. However, it seems to be an important protein in the construction of the chitin–protein–mineral complex of the gastroliths of *C. quadricarinatus*, since it is found both in the Sol and Ins profiles. In addition, the protein is detected as being heavily phosphorylated and as a strong calcium-binder, especially the fraction extracted in the Ins, which is also considered to be chitin-binding and was shown to play part in *in vitro* precipitation and stabilization of ACC.

CqCDA1 is a deduced protein, whose transcript sequence was obtained from a gastrolith-forming tissue cDNA library following a microarray experiment in which it was identified as highly up-regulated in the tissue during premolt (Yudkovski et al. 2010). The protein sequence contains, in addition to a signal sequence, ChtBD2 and chitin deacetylase (CDA) domains, making it a possible chitin-binder.

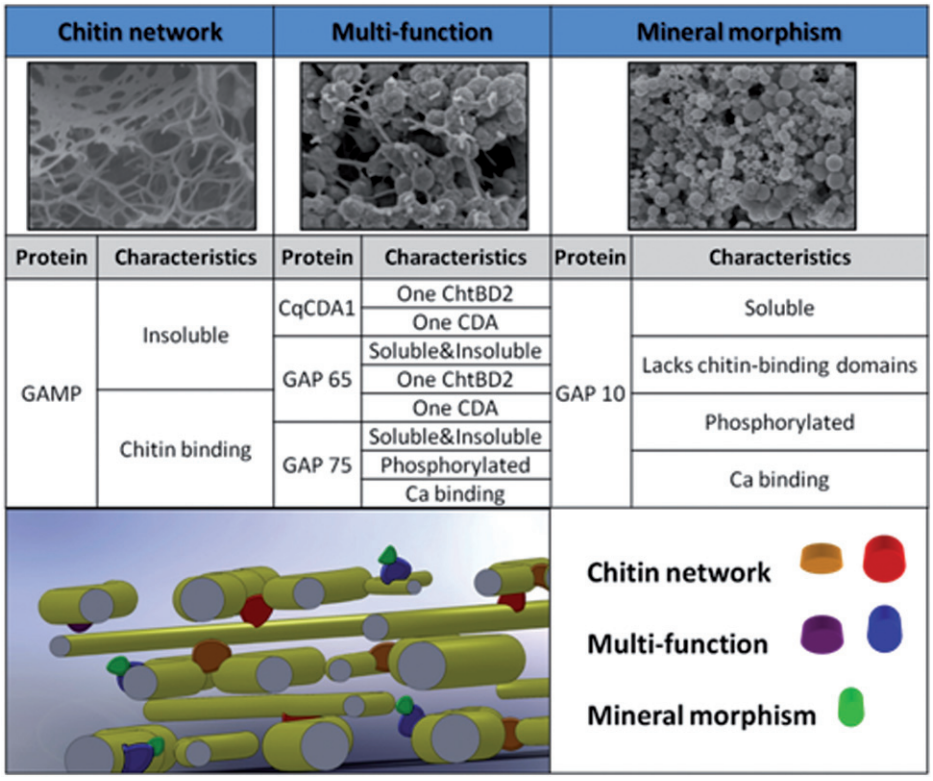

Figure 7. A theoretical model for different modes of involvement of proteins in the assembly of the gastrolith chitin–protein–mineral complex. Upper table – assignment of known GAPs to three structurally functional groups. Lower panel – schematic model illustrating the hypothesized association between the different proteins and the chitin fibers [the model was taken with permission from Gafni (2010)].

CqCDA1 also contains the same type of long C-terminal stretch as found in GAP 65.

Protein involvement in gastrolith assembly – summary

To gain a better understanding of the involvement of proteins in the assembly of the extracellular matrix of gastroliths and its different structural aspects, we have divided the GAPs into three hypothetical groups (Figure 7). The first group consists of proteins that are exclusively involved in the construction of the chitinous matrix (Figure 7, table, left panel). These proteins play various roles in the formation of the chitin–protein fibers and network that make up the gastrolith thin lamellas. The second group consists of proteins that are directly involved in the precipitation of the ACC and that have no direct association with chitin (Figure 7, table, right panel). The third group consists of those proteins that have roles in both processes, and are therefore considered multi-task players (Figure 7, table, middle panel).

To date, five proteins have been identified from crayfish gastroliths: GAMP from *P. clarkii* and GAP 65, CqCDA1, GAP 75, and GAP 10 from *C. quadricarinatus*. The first four proteins are believed to be chitin-binding proteins, because of the presence of ChtBDs in their putative sequences, as is the case for GAP 65 and CqCDA1, or because of their strong presence in the EGTA-insoluble protein profile of the gastroliths, as is the case for GAMP, GAP 75, and for GAP 65. Assigning chitin-binding properties to these proteins implies their involvement in the formation and arrangement of the chitinous matrix of the gastroliths, as shown by gene silencing for *GAP 65* (Shechter et al. 2008b). However, we hypothesize that these proteins are more than just chitin-binders, rather they are considered multi-task players, possessing additional roles in protein–protein interaction and/or the precipitation of ACC. This hypothesis is based, in part, on the *in vitro* calcium carbonate precipitation assays conducted with GAMP and GAP 65. Moreover, GAP 65 and CqCDA1 are both members of a family of cuticular proteins that also found in insects (Dixit et al. 2008), since they both contain the ChtBD2 and chitin/polysaccharide-deacetylase domains, in addition to a yet uncharacterized C-terminal domain (Shechter et al. 2008b; Yudkovski et al. 2010). This homology between GAP 65 and CqCDA1 makes the two proteins candidates for the group of multi-task players (Figure 7, model, blue and purple shapes). GAP 75,

in addition to its strong association with the chitin matrix – requiring the use of 6 M urea for its full extraction – was found both to be strongly phosphorylated and to have a calcium-binding ability (Bentov et al. 2010). The last two characteristics are associated with calcium deposition and may influence the polymorph of the calcium carbonate precipitate in the gastroliths. Therefore, GAP 75 is also suggested to be one of the multi-task proteins of the gastroliths (Figure 7, model, blue and purple shapes).

In the present model, we assigned GAMP to the chitin-structure-related proteins (Figure 7, model, red and orange shapes) and not to the multi-task proteins, because of the strong indications that its involvement in the matrix, possibly as a part of the chitin–protein fiber arrangement, is its primary function. To date, there is no protein that has been identified as exclusively matrix related and involved solely in the network formation, although a family of such proteins is known in insects; these proteins contain sets of one or more ChtBDs of the type ChtBD2 (Jasrapuria et al. 2010). We have thus assumed the presence of several such proteins in our suggested model, as indicated in Figure 7 by the red and orange shapes.

GAP 10 is not a chitin-binding protein; however, it is phosphorylated and calcium-binding (Glazer et al. 2010). Gene silencing of *GAP 10* resulted in a considerable delay in premolt duration and the development of gastroliths with significant surface irregularities. The above data suggest significant involvement of the protein in the formation of the chitin–protein–mineral complex of the gastrolith, especially with regard to the deposition of calcium carbonate (Figure 7, model, green shapes). Since there is no indication of direct association between GAP 10 and the chitin matrix, we have previously hypothesized that it is somehow attached through protein–protein interaction via one of the multi-task chitin-binding players – either known or as yet undiscovered (Glazer et al. 2010).

In conclusion, in the past few years, there have been significant contributions to the study of gastroliths, both to the organic matrix aspect and to the mineral aspect. Gastroliths can be considered as a relatively simple extracellular model for the study of mineralized matrices in a biological setting. They are constructed of concentric layers, all built of a similar combination of organic matrix and calcium carbonate, as opposed to crustacean calcified cuticles, which consist of four very different layers and additional elements such as pigmentation. Since gastroliths are extracellular matrices, it is believed that their construction involves a manageable set of proteins and other macromolecules and additives. In this article, we presented our first hypothetical model for the involvement of proteins in gastrolith assembly. This model is still in a preliminary stage, calling for the discovery of more proteins and additional functional assays to enable us to reach a more comprehensive and complete picture of this unique system.

Acknowledgments

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